The impact of wine closure and packaging type, and light and temperature exposure on the concentration of 3-alkyl-2-methoxypyrazines and other key constituents of wine

By
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A Thesis

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Abstract

3-alkyl-2-methoxypyrazines (MPs) are grape- and insect-derived odor-active compounds responsible for vegetative percepts that are detrimental to wine quality when elevated. This study tested both the effect of closure/packaging types and light/temperature storage conditions on MPs (isopropyl-, *sec*butyl-, and isobutyl-MP) in wine. An MP-enriched wine rapidly (after 140 hours) and significantly decreased in MP concentration after natural and synthetic cork contact (immersion of closures in wine). This decrease was greatest with synthetic closures (70% - 89% reduction) and *sec*butyl-MP. Subsequently storage trials tested the effects of commercial closure/packaging options (natural cork, agglomerate cork, synthetic corks, screwcaps and TetraPak® cartons) on MPs in MP-enriched Riesling and Cabernet Franc over 18 months. Regardless of packaging, isobutyl-MP was the most altered from bottling. Notably, all MP levels tended to decrease to the greatest extent in TetraPak® cartons (~34% for all MPs) and there was evidence of contribution of isoproyl- and *sec*butyl-MP from cork-based closures (i.e. ~30% increase in *sec*butyl-MP after 6 months) or from an unidentified wine constituent. To test the effects of various light/temperature conditions (light exposed at ambient temperature in three different bottle hues, light excluded at ambient temperature and light excluded at a "cellar" temperature (14°C)), MP-enriched Riesling and Cabernet Franc were also analyzed for MP concentrations over 12 months. MPs did not vary consistently with light or temperature. Other odorants and physico-chemical properties were tested in all wines during storage trials and closely agree with previous literature. These results provide novel insights into MPs during ageing, interactions with packaging and storage conditions, and assist in the selection of storage conditions/packaging for optimal wine quality.

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List of Symbols, Nomenclature and Abbreviations

Abbreviation	Expansion
Agl	agglomerate cork
amber	light, amber hue bottle, and ambient temperature
CDensity	wine colour density
clear	light, clear hue bottle, and ambient temperature
dark + ambient temp	dark and ambient temperature treatment
dark + cellar	dark and cellar treatment
EtCap	ethyl caprate
EtCapryl	ethyl caprylate
EtHex	ethyl hexanoate
FS	flavour scavenging
GC	gas chromatography
GC-FID	gas chromatography-flame ionisation detection
GC-MS	gas chromatography-mass spectrometry
green	light, green hue bottle, and ambient temperature
HA	*Harmonia axyridis*
HPLC	high performance liquid chromatography
HS-SPME	headspace-solidphase-microextraction
IBMP	3-isobutyl-2-methoxypyrazine
IPMP	3-isopropyl-2-methoxypyrazine
IsoAce	isoamyl acetate
LBT	ladybug taint
MLF	malolactic fermentation
MP(s)	3-alkyl-2-methoxypyrazine(s)
MSD	mass selective detector
NatC	natural cork
OctAcid	octanoic acid
PCA	principal components analysis
PE	polyethylene
PheAce	phenethyl acetate
PheEtOH	phenyl ethanol
RedPig	degree of red pigmentation
RSD	relative standard deviation
SBMP	3-*sec*butyl-2-methoxypyrazine
Scap	screwcap
Syn-Ex	extruded synthetic closure
Syn-M	moulded synthetic closure
Tpk	Tetrapak® cartons

Thesis Outline

This thesis consists of four chapters; Chapter 1 is the background literature review, Chapters 2 and 3 are research trials that are formatted for submission to scientific journals, and Chapter 4 is a final, synopsis chapter, which summarizes the previous sections and ties all conclusions together in terms of overall significance and further research recommendations. Appendices A and B contain supplementary data and a preliminary research trial (Preliminary Trial) that is also formatted in manuscript-style for submission to a scientific journal, respectively.

Contribution of candidate to Chapters 2, 3 and Preliminary Trial

The body of this thesis is a compilation of peer-reviewed papers with multiple authors and hence to follow is a summary of the work done by the candidate. The candidate completed all experimental work in Chapters 2 and 3 and the Preliminary Trial, with the following exception, the quantification of methoxypyrazine compounds in the Preliminary Trial was completed at the LCBO – Quality Assurance laboratory. The candidate completed all statistical analysis and wrote the content in Chapters 2 and 3. The candidate was assisted in writing of the Preliminary Trial paper by the thesis supervisor. Because the thesis is a collection of multi-authored manuscripts, the candidate's supervisor also contributed minor editing for the final drafts and the co-authors contributed to the study design, data interpretation and chapter formatting.

Chapter 1

Literature Review

I. 3-Alkyl-2-Methoxypyrazines in Grapes and Wine

i. Sources

Wine aroma is formed by the intricate balance of several hundred compounds (1). These volatile compounds are grouped into categories, such as "fruity" or "floral", based on common perceptions. One important class of wine aromas is termed "vegetative/herbaceous", which includes percepts such as "bell pepper", "asparagus", and "grassy". Vegetative aromas are critical constituents of wine aroma (2, 3). They are elicited by two chemical classes of compounds, the 3-alkyl-2-methoxypyrazines (MPs) and the aliphatic carbonyls. These compound classes vary in chemical structure, aroma quality and relative importance in grapes and wines.

The MPs that contribute to greenness in wines are nitrogen-containing heterocyclic ring structures, with alkyl side groups, known as the 3-alkyl-2-methoxypyrazines. Aliphatic carbonyls include alcohols, acids, aldehydes and ketones with a 6-carbon backbone:

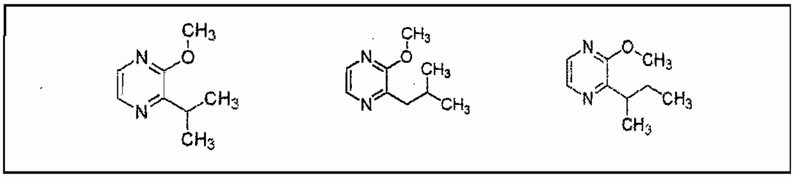

Figure 1-1: (L-R): 3-isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxypyrazine (IBMP) and 3-*sec*butyl-2-methoxypyrazine (SBMP)

Vegetal aromas in wine described as "green pepper", "earthy", and "musty" are due to MP compounds (4), while more "fresh" aromas like "cucumber", "grass", "green wood" and "lemon" are due to the carbonyl compounds (5).

MPs are important constituents of wine flavour because of special sensory attributes. MPs can cause a perceived decrease in intensity, or "masking", of other aromas and also can define the varietal character in certain wines and wine-styles (2, 3). MPs present in nature possess an intense and unique aroma-potency; they are interesting or attractive at low concentrations, but at slightly higher levels become very characteristic and repellent (6). Interestingly, this is also observed in wine; elevated MPs can be overpowering and negative to wine aroma (7). Their significance is additionally evidenced by their prevalence throughout the natural world. They occur in a wide survey of raw vegetables (8) and other food products (9), are produced by microorganisms (10, 11) and as pheromonal compounds by insects and plant species (12, 13) likely as warning compounds to repel predators (14).

MPs occur in wine from two main sources, reviewed below. Primarily MPs are grape-derived, however recently a secondary source has been identified, the inadvertent incorporation of the ladybug *Harmonia axyridis* with a harvest and fermentation, known as ladybug taint (LBT; (15)).

MPs are secondary metabolites produced in grapes. Although MP biosynthesis in grapes has not been fully characterized, the final step of a synthesis method proposed in raw vegetables (8) has been shown to exist in *Vitis vinifera* grapes (16). MP synthesis and accumulation occurs before the ripening stage of fruit growth (17) and decreases thereafter (17-20) implying that MPs might act as a deterrent from the consumption of unripe fruit, when the seeds are too immature for survival (21). Most *Vitis vinifera* grape varieties contain MPs (17), however they are present in higher concentration and often able to impact aroma, particularly of varieties from Bordeaux, France; Sauvignon Blanc (2, 19) Cabernet Sauvignon, Merlot and/or Cabernet Franc (7, 17, 20, 22, 23) and Carmenere (24). Greenness, from inherent MPs can positively add to the varietal character for some of these wines, i.e., Marlborough Sauvignon Blanc (3). MPs are present at relatively elevated concentrations in grapes in cooler climates (7, 19, 20, 23, 25), or under-ripe fruit (17, 23), a trend consistent with "vegetative" aroma intensity (26). This is presumed to be a result of a decreased amount of sunlight exposure (17). Therefore, at elevated concentrations, MP-mediated greenness in wine is inversely related with optimal grape ripeness and consequently associated with poor wine quality (23). This distinction is complex and is not universally definable.

Ladybug-derived MPs, specifically IPMP, in wines have recently been identified as the causal compound(s) of LBT (15, 27). Anecdotal evidence from grape growers linked the presence of great numbers of ladybugs in vineyards at harvest time with atypical aromas consistent with MPs (beginning in 2001, in Southern Ontario). The beetles were identified as the non-native coccinellid species *Harmonia axyridis* (HA; commonly known as the Multicoloured Asian Lady Beetle). HA has many coloured and spotted morphs dependent on many factors, reviewed in (28), but can be identified by four black spots on the pronotum, which loosely form a letter "M" or "W", (***Figure 1-2***). The 2001 vintage in Southern Ontario was greatly affected by HA-tainted wines and represents a substantial loss for the local wine industry (29).

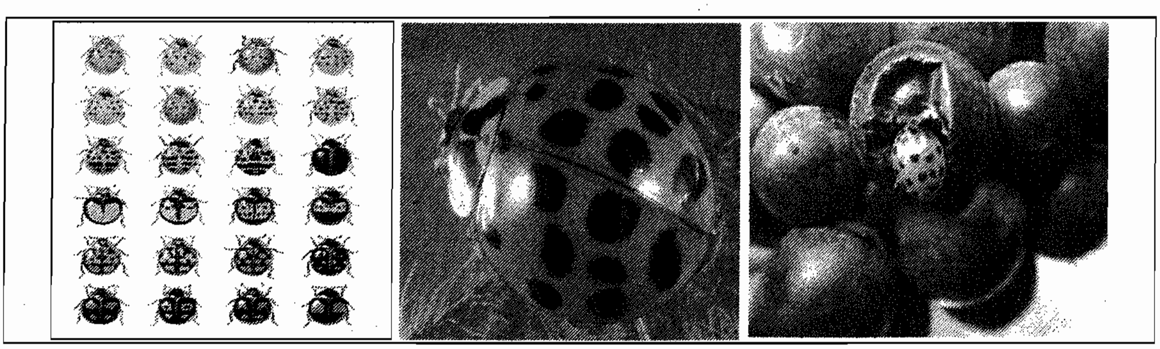

Figure 1-2: (L-R): Harmonia axyridis (HA) colour morphs (30), HA close up (31), and HA feeding on grapes (32).

HA was successfully introduced to North America from Eastern Asia in the 1980s as a biological control agent to prey on aphid species (28). As recently reviewed, HA have been extremely successful at competing with native species due to a lack of dietary specificity, high reproductive capacity, voracious predation and quick adaptation to a broad range of climates (33). HA have become the most predominant aphidophagous coccinellid in many areas of North America in less than twenty years (34). Due to their aroma potency, MPs (especially IPMP) act as warning signals in many aposematic insects (6), including HA (35) which can emit haemolymph (containing MPs) as a fear response termed "reflex bleeding" (36). Wines fermented in the presence of HA show an increase in MPs, mostly IPMP, while other wine chemistry parameters are more or less unchanged (15). Additionally, vinification in the presence of increasing concentrations of HA shows an increase in the characteristics of LBT; including higher intensities of aromas and flavours of "peanut", "bell pepper" and "asparagus" in a white wine and "peanut", "asparagus/bell pepper" and "earthy/herbaceous" in red wine and a general decrease in "fruity/floral" aroma intensities in both red and white wine (37). LBT is relatively stable in wines during ageing (15).

Regardless of source, elevated MP concentration can be detrimental to wine quality and MPs in wine could require remediation. However MP management is complicated. The main issues affecting MP control in wine are their trace concentration, extremely low odour detection thresholds and challenges in quantification.

ii. Concentration

Compared with the majority of wine aroma volatiles, MPs are present in trace quantities. Most wine volatiles are in the mg/L or μg/L range (38, 39) while MPs are present in the low ng/L range, regardless of source (15, 19).

The dominant MP found in grape varieties is IBMP followed by IPMP and then SBMP. A wide survey of Sauvignon Blanc grapes and wines found consistent ratios between grapes and resultant wines, which had concentration ranges of IBMP, IPMP and SBMP, respectively of 0.6-38.1 ng/L, 0.9-5.6 ng/L and 0.1-1.3 ng/L (19). These relative abundances are consistent for red grapes (40) and wines (2, 23). Although SBMP and IPMP are often found in very low concentration, their possible role in the modification of aroma should not be discounted (2). Within grapes clusters, IBMP is present in the greatest amount in the stems followed by skins and seeds of grapes (22).

In the case of HA-contributed MPs, the relative ratios are different to those inherently present in grapes. The haemolymph of *Harmonia axyridis* contains IPMP, IBMP and SBMP at approximately 27.5μg/bug, 3.2μg/bug and 2.6μg/bug, respectively (35). Qualitative analysis on a live HA beetle confirmed these approximate ratios (41). IPMP is the most abundant MP in ladybugs (35) and the causal compound for LBT in wines (15, 27). Experimental wine fermented with 10 HA beetles/litre increased in IPMP and IBMP by approximately 29ng/L and 6ng/L, respectively, (15) and commercial wines rejected by the Vintners Quality Alliance of Ontario for LBT had approximately 50ng/L of IPMP and negligible amounts of other MPs (Pickering, G., pers. comm).

iii. Threshold

Detection thresholds, the concentration at which an aroma is detected, indicate the aromatic-potency of volatile compounds. Typically, detection thresholds for wine volatiles are in parts per million (mg/L) or parts per billion (μg/L) (38). MPs, however, possess distinctly low thresholds, therefore even at very low concentrations they can contribute to or dominate aroma. The detection threshold for IBMP is approximately 10 ng/L (23) in red wines. The detection threshold for IPMP in red wine is between 2 ng/L (4) to 1.03 ng/L and between 0.32 and 1.56

ng/L in white wines (42). There is no published data on SBMP thresholds in wine, however SBMP odour thresholds in water are comparable to the other MPs as reviewed in Sala *et al.* (43).

iv. Analysis

Trace volatile analysis can be extremely difficult and requires a high level of instrumental sensitivity. First identified in Cabernet Sauvignon grapes in 1975, IBMP was tentatively linked to the vegetative character of Cabernet Sauvignon wines, however this first effort was qualitative and unable to describe the sensory impact of IBMP (44). Gas chromatography-mass spectrometry (GC-MS) technology and the use of liquid-liquid extraction with a deuterium-labeled analog of IBMP has allowed for the isolation and quantification of MPs in wine at or below their detection threshold (44, 45, 7, 20). Only recently has the presence and role of MPs in grapes and wines been characterized (19).

In general, techniques involving liquid-liquid extractions are overly laborious and time-consuming (46). Methods have been developed which employ solid-phase extraction with GC-MS (15) and HS-SPME with gas chromatography-nitrogen-phosphorus detection (GC-NPD) (40, 46) without the use of isotopically labelled internal standards, and consequently suffered from insensitivity problems (41). A recent method with extremely high sensitivity, accuracy and precision has been developed and validated that involves the optimization of matrix parameters and quantification HS-SPME-GC-MS and deuterated analogs for all target analytes (IBMP, SBMP and IPMP) (41).

The mediation of MPs in wine is crucial to preserving wine quality. Control efforts include both prevention and remediation- and in terms of grapes and wines, viticultural and oenological processes, respectively. Mediation of MP through viticultural and oenological practices are reviewed below.

II. Mediation Practices

i. Viticultural

The prevention of elevated MPs in wine is the preferred method of control (29) and includes both viticultural practices that alter grape ripening and exclude HA from vineyards at harvest.

MPs present naturally in grapes are synthesized before the ripening stage of fruit growth (veraison) (17) and decrease thereafter (17-20). IPMP and SBMP increase in concentration slightly later than IBMP, which does so before veraison, but more severely resulting in similar final concentration for all MPs at physiological ripeness (47). Others have corroborated this, and noted that IPMP concentration is less influenced by seasonal climatic factors than IBMP (24). MP degradation after veraison is presumably due to non-enzymatic photo-decomposition of MPs in sunlight (17); however, others have found conflicting data regarding this phenomenon (18, 47). Generally, MP mediation during grape-growing involve manipulating fruit light exposure; by minimizing the fruit shading from the vegetative canopy by non-irrigation of vines, decreasing planting density (43), and varying vine training systems (47).

Solutions to MPs derived from HA beetles have focused on the exclusion of HA from the vineyard. HA appear in vineyards in large numbers around the time of harvest due to their aggregation and over-wintering behaviour (48) in connection with damaged, autumn-ripening fruit. However, HA are not inflicting damage (49), i.e., grape berry injury or splitting (48). Exclusion strategies were recently reviewed (50) and include the use of potential pheromonal lures and pesticide sprays, however, because of pre-harvest intervals (the amount of time after a spray treatment that one may harvest) and the late-season influx of HA, pesticide control is currently limited to only three spray products in Canada. The use of pesticide sprays often allows for dead ladybeetles, still present on grape clusters to become incorporated into a fermentation, but recent research on HA has observed no increase in LBT characteristics or IPMP concentration $\geq$3 days post-mortem (27). An indirect method for decreasing the presence of beetles focuses on decreasing berry damage (48). Continued work is needed in this area as thresholds suggest that even a small number of HA in the vineyard can negatively impact wine quality. This threshold is approximately 1 beetle per vine, or 200-400 beetles per tonne of grapes, recommended as a conservative, "safe" threshold for grape growers (50) or 0.27 beetle per grape cluster of Frontenac based on a threshold at which 10% of the population can detect an off-flavour (51).

ii. Oenological

During wine fermentation many new volatiles are produced and lost, however, MPs are predominantly stable (22, 52) even after a malolactic fermentation (47). IBMP content does increase after one day of maceration (contact with skins) in red wine fermentation (22), unlike IPMP and SBMP, confirming that IBMP is present in grape skins in greater amounts than other MPs (22, 47). HA-derived MPs are imparted to wine only after ladybugs are included in the first oenological processing stage (crushing/destemming) and not before (i.e., during mechanical harvest or vineyard habitation) (53). MP content is substantially reduced (~50% decrease) with pre-fermentation practices of settling white juice (to promote the flocculation and remove heavier matter) for IBMP (22) and IPMP (41), bentonite clarification also significantly decreases IPMP content of white wine (41), and thermo-vinification (the process of heating juice to ~60-80°C for a short period prior to fermentation to increase phenolic extraction) can drastically reduce IBMP content in red wine (54). Unfortunately these practices are not always appropriate or feasible, given winemaking constraints and wine-style limitations. Following the hypothesis that yeast cells may metabolize MPs as a nitrogen source, a recent study tested various commercial yeast strains for the ability to alter wine MP content and found that while most common strains (EC 1118, D80, DV10) had no effect on MPs, one strain, BM45, contributed about 30% of IPMP to final wine (55).

Post-fermentation, oenological techniques that are traditionally used to alter aroma, i.e., bentonite, activated charcoal, oak chips, were tested for effects on MPs and sensory characteristics of wines. Researchers found that IPMP concentration was lowered by activated charcoal in white wine and de-odourized oak in red wine, but the sensory characteristics of MPs were generally unaffected, except under treatment with oak chips (29). Additionally, these treatments may not be appropriate for wine style and other wine parameters.

As MP concentration is largely unaffected by normal winemaking practices, other post-fermentation practices deserve further exploration for potential to affect MP concentrations. Packaging and closure options are worth considering in this regard.

III. Wine Closure and Packaging

An ideal food packaging system will preserve a consumable's freshness (56), however with wine this objective is not as simple. Wines are in constant flux; after packaging its chemical and sensory attributes continue to change. Perhaps the most important of these changes is flavour modification. The volatile constituents of wine are transformed during bottle or barrel ageing by oxidation or reduction reactions to form a "bouquet", as reviewed in (1). Flavour compounds are transformed and created in ageing wines due to both intrinsic processes occurring within the wine matrix itself and extrinsic processes occurring from the surrounding environment (57), the latter greatly influenced by packaging. Flavour modification related to packaging includes three major mechanisms: the indirect mechanism of gas movement (or permeation), and the direct movement of flavour compounds into a wine from its packaging (migration) or vice versa (sorption; ***Figure 1-3***).

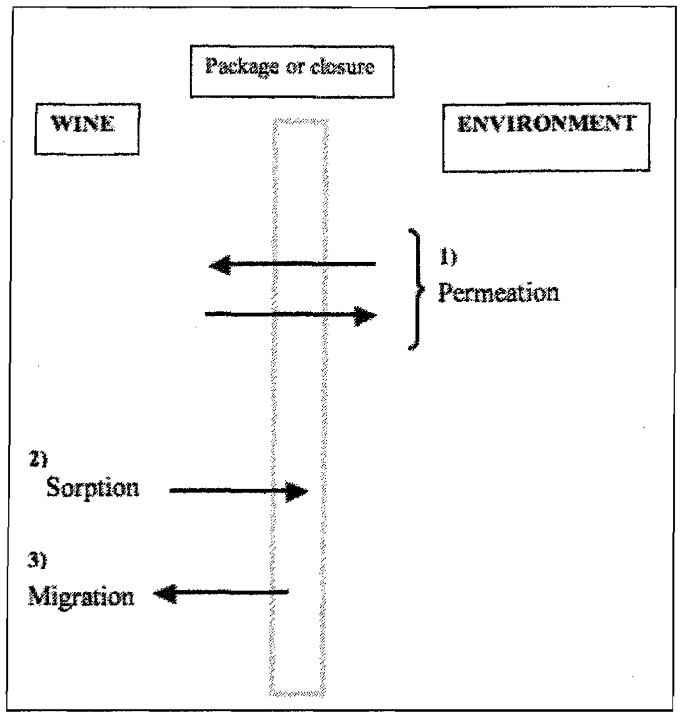

Fig 1-3: Wine, environment and packaging interactions. Modification of (58)

The wine quality regulating body in Ontario, the Vintner's Quality Alliance of Ontario (VQA-O), has approved a variety of closure and packaging options including natural corks, natural cork variations, synthetic corks, screw-caps with glass bottles. Additionally, TetraPak® cartons are used for non-VQA-O products and international products. These closure and packaging options greatly affect wine ageing processes and are reviewed below.

i. Closures

Natural cork has dominated as a wine closure for over 200 years due to its many benefits; it is flexible, compressible, relatively impermeable to air and liquid, and chemically and microbiologically inert (59). But natural cork closures are imperfect. Most notoriously, natural corks can contribute an aroma defect known as “cork taint” to wines, affecting between 2-15% of wines, that has been well studied and reviewed (60, 61). Cork taint is due to compounds, such as trichloroanisoles (TCA), that are produced by fungal strains present in cork material metabolizing residual chlorophenols that are produced after the sterilization ("bleaching") of cork planks, and is characterized by “mouldy”, “musty/wet” flavours and the muting of desirable “fruity/floral” aromas (60). Natural corks have also been implicated in the sporadic oxidative spoilage of wine (62), in which some corks allow an increased amount of oxygen to permeate into wine. Finally, natural cork is associated with high costs. For example, a wine industry such as Australia, California and South Africa must deal with costs associated with importation from Western Europe (63). In response to these issues, alternative packaging options have been developed.

Less costly cork-based alternatives, which include composite cork and colmate cork, are produced by joining small fragments of natural cork together at high pressures with glue, and by filling natural cork pores with fine cork dust and glue, respectively. Developed in response to all the problems associated with natural cork and providing a microbiologically inert stopper are the synthetic closures (63). Moulded synthetic corks consist of a silicon coating and internal foam and extruded synthetic corks consist of a foam interior and stiff outer layer. “Technical closures” combine 1 natural corks disks at either end (“1+1”), 2 natural cork disks at either end (“2+2”), or 2 natural cork disks at one end only (“2+0”) of an agglomerate cork body. The screw cap closure (or “roll-on-tamper-evident”; ROTE) is an aluminum-covered capsule with various inert liners that contact the wine. (***Figure 1-4***).

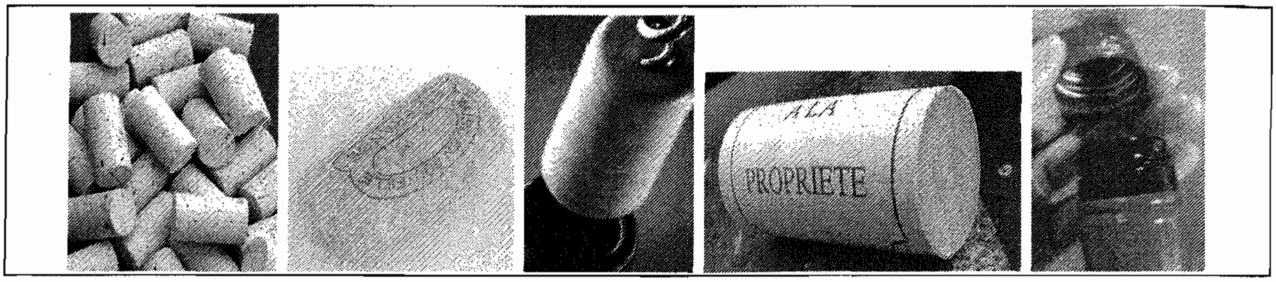

Figure 1-4: Closures: (L-R): natural corks (64), composite cork (65), a moulded synthetic cork (66), an extruded synthetic cork (67) and a screw cap closure (68)

ii. Packaging

Wines have been packaged in glass bottles for centuries. Various glass bottle hues are available and able to affect flavour during ageing as a result of differing light transmittance. Light transmittance is the amount of light from any source that can pass through a material without being absorbed, often quantified as a percentage. Glass bottles are available in three main hues: green, clear (flint) and amber (brown). Wavelengths in the UV spectrum and the blue portion of the visible spectrum (~350nm-550nm) produce negative effects on the quality of wines as reviewed in (69). Bottle transmittance at various wavelengths. Overall, it is recognized that amber bottles absorb all wavelengths below 550nm, green hue bottles are intermediate in absorption and clear hue bottles allow all visible wavelengths to pass (69). A wide range of bottle hues exists, including "emerald green", "antique green", "french green", "Georgia green", "cobalt blue", "flint" and "amber" displays the same overall trend (70).

Light exposure can increase white wine browning and "oxidized" aromas, however, this process is not directly related to phenolic compounds and the underlying actions are not yet understood (71). There is scarce literature on the participation of light on the volatile content of wines, but it is cited to have a negligible effect on some Sherry wine aroma compounds over time (71). However, light exposure can trigger the development of so-called "light-struck" aromas, from photochemical reactions, involving the reduction of riboflavin (vitamin B2) and subsequent decomposition of ester compounds and/or production of volatile sulphur compounds from sulphur-containing amino acids (72). Light-struck aromas are produced in wines after a relatively short-term exposure to fluorescent light and observed to decrease citrus aroma intensity, and increase the intensity of "cooked cabbage", "corn nuts", "wet dog/wet wool", "soy/marmite" aromas in Champagne and base wines (73). Light exposure decreases MPs in grapes ripening in vineyards (18) and simple photo-decomposition may occur for MPs in wines bottled in hues that allow for increased light transmittance. This effect has been observed in a preliminary study reported in a conference proceeding (4), but surprisingly not followed up in the peer-reviewed literature.

Alternatives to glass bottles have been developed to improve costs and are largely intended for short-term wine ageing. Bottling alternatives include TetraPak® cartons, "Bag-in-box", and most recently PET bottles and aluminum cans (***Figure 1-6***). TetraPak® cartons used for wine packaging are often aseptic, multilayer carton that consist of (outer→inner): a polyethylene layer (PE; to protect against moisture from atmosphere), paper layer (for stability), PE layer (for adhesion), aluminum foil (for a barrier to oxygen, light and flavour volatiles), PE (for adhesion), PE (to seal in liquid).

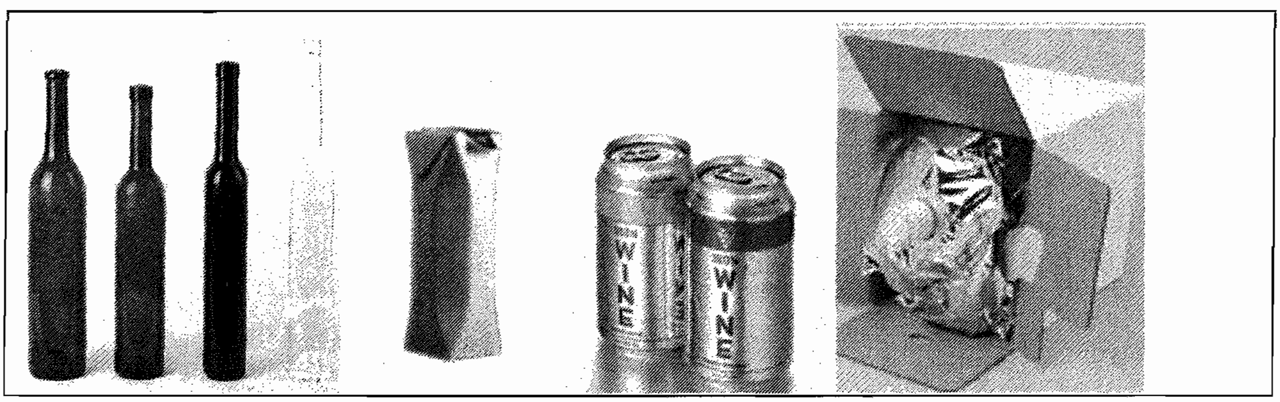

Figure 1-5: Packages (LtoR): glass bottles of various hues (74), a TetraPak® carton (75), aluminum cans (76), "bag-in-box" package (77)

Many of the described closure/packaging options for wine have been investigated for their potential for flavour modification and are reviewed below.

iii. Flavour Modification

a. Permeation (gas movement)

Gas movement can greatly influence flavour compounds. In general, effective packaging acts as a barrier to contact with air, i.e., to prevent evaporation (56), however, packaging options in wines allow varying degrees of gas permeation (78). Gas permeation through packaging is a complex issue and has been dubbed "the new argument in the closures debate" (79). Minimal oxygen ingress is integral to the development of red wine flavour profile, promoting phenolic reactions that reduce astringency and soften colour, as summarized in (67), but is generally regarded as negative to white wine quality (81). At elevated levels and in certain wines the negative effects of oxygen ingress include a decrease in free SO_2 concentration (82), a decrease in "fruity" intensity, an increase in 'oxidation' sensory rating (83), an increase in browning (81, 83) and a decrease in overall wine quality (83). Conversely, wines that are stored in the closures that allow little to no gas permeation (screwcaps) can be associated with negative "reduced" (or sulphurous) aromas (84)- although oenological practices can aid in avoiding the development of these aromas (83). Overall the optimal permeability of packaging depends on the wine and the desired product attributes, including flavour, colour and mouth-feel.

As evidenced in the following literature, oxygen permeability is dependent on closure/packaging type during storage. Over 20 months, synthetic closures allowed the greatest amount of oxygen permeation (~9 mg/L), natural cork stoppers were intermediate (3.5-4.5 mg/L), and screw cap closures were the most impermeable to O_2 movement (~1.5 mg/L) (80).

The route of oxygen ingress also varies between closures; natural cork releases air from inside the closure for approximately 6 months, and thereafter allows a small amount of atmospheric oxygen to enter wine, synthetic cork allows atmospheric oxygen to enter wine immediately and to a greater extent, and screwcap provides an absolute barrier to atmospheric gas (78). This trend is supported in other studies (84), while synthetic stoppers made of expanded polyethylene or with diaphragm layers provide similar oxygen permeability to natural corks (63). TetraPak® cartons can provide protection against gas permeation to a similar extent as glass bottles, up to about 12 months (85).

Gas permeation through wine packaging is relatively slow and may not manifest itself in perceivable changes, like browning, in the first year of packaging, as has been seen in previous research (85). This approximate timeline is the intended upper limit for consumption of many wines finished in alternative packages (86) and industry statisticians report that, globally, 95% of wine is consumed within 18 months of bottling and 99% of wine is consumed within 24 hours of purchase (87). The direct effect of packaging material on wine flavour compounds occurs within a shorter period of time as seen in (88) and could thus be of more importance to wine quality.

b. Flavour Scavenging

Distinct from changes occurring within the wine matrix and gas-mediated changes, there are also flavour modifications due to direct contact with packaging. These can result from the migration of flavour compounds into or out of the wine media, are often associated with a loss of quality, and are known as "flavour scalping" or "flavour scavenging" (FS; 89). Food science research has elucidated the main processes occurring in compound migration and attempted to predict FS. Largely, FS research has focused on the sorption of d-limonene in citrus juice into polymer packaging (58, 90). FS processes involve the movement of volatiles and can be summarized under two main events: partition and diffusion (91).

Partitioning is under the control of a rate estimator known as the partition coefficient (K) and is influenced by the compound's characteristics (e.g., its concentration and boiling point), the polymer characteristics (e.g., the surface area and polarity), and external factors (e.g., the pH and storage temperature) (89).

Diffusion events are often quantified or predicted using variations of Fick's first law: $M_x/\Delta t*L=P*A*\Delta p_x$ (where $M_x/\Delta t$ = the transport rate of material "X", through film area "A", of thickness "L", chemical potential by pressure difference "Δpx", P= permeability coefficient) and begin with collision of the penetrant molecule with the package or liquid, followed by sorption into the media or material through the matrix (89).

Modeling studies have attempted to predict the sorption behaviour of flavour compounds and packaging, including oxygen permeability of polymer packaging (92). However, no modeling system has been able to accurately account for all the factors that can influence FS (89). Although FS studies have only recently been conducted on wine and packaging, there have been major findings in the last decade implicating both closure and packaging material in the migration and sorption of volatiles.

Migration

FS studies on the migration of volatiles from closure or packaging into wines have largely focused on cork taint. Chloroanisoles (mainly 2,4,6-trichloroanisole), chlorophenols (mainly pentachlorophenol), and guiaicol have all been found to contribute to cork taint after migration from contaminated natural cork closure into wine (60, 61, 93). However, in a wide survey of commercial wines affected with cork taint, trichloroanisoles and trichlorophenols were found to only be minor constituents (94) suggesting other compounds are implicated.

Although less common, other volatiles can migrate from closure to wine. This is observed with lead from eroded tin capsules (95) and 2-methoxy-3,5-dimethylpyrazine (MDMP) from fungally-infected natural corks (96). Others have noted a "glue-like" aroma in wines closed with synthetic corks (both moulded and extruded examples) (83), but the corresponding compound(s) have not been identified and this effect remains somewhat elusive (84). Additionally, the volatile content of cork bark, new cork stoppers and used cork stoppers include over 80 compounds (97) suggesting closures have a great ability to impart volatiles to wine.

Sorption

FS sorption has only recently been studied in wine. Starting in 1999, a group of researchers looking into the ability of natural corks to impart compounds to wine noted the elevated ability of natural cork to absorb compounds, which they recognized as FS (98). Sorption can be measured in wine studies both directly (i.e., by quantifying a volatile in a packaging material) or indirectly (i.e., by quantifying the concentration change in wine before and after contact with packaging material) (88). A longitudinal study completed at the Australian Wine Research Institute showed that FS of wine volatiles by closures is selective and differential; synthetic closures had the greatest ability to decrease the concentration of volatiles, followed by technical corks and natural corks, while screw caps were unable to affect volatile concentration. Relatively non-polar volatiles were the most affected by FS from closures (88). These findings have been mirrored in other studies (63, 82, 99). Although TetraPak® packaging has not been investigated for its FS abilities in published wine studies, the polyethylene layers of tetrapak packaging have been strongly connected with flavour sorption in other foodstuffs, such as citrus juice (100). This suggests that FS may be of significant importance for wine volatiles and the many packaging options available, and should be examined for ability to alter MP concentration in wine.

IV. Conclusion

3-alkyl-2-methoxypyrazines are aromatically unique and potentially potent vegetative compounds in wine. Although they can contribute positively to wine quality, more often, they are associated with under-ripe, low-quality fruit and ladybug taint. Although the prevention of elevated MPs would be preferable to remediation, elevated MPs often necessitate oenological remediation to preserve wine quality. The control of MPs in wine is complicated by their low concentrations, and odour thresholds, and the challenges of complex analytical techniques.

A review of wine closure and packaging research reveals two approaches that may affect MPs in wine; FS through closure and packaging contact and photo-degradation from increased light transmittance through glass bottles. Food technology research has focused on the phenomenon of FS, where volatiles can be absorbed into packaging material. Modern oenological closure and packaging options include a wide variety of natural and polymer materials, some of which have not been examined for FS capabilities and none of which have been comprehensively studied for effects on MPs. MPs can be photo-degraded in grapes while ripening in the vineyard (17) and it may follow that MPs in wine could be decomposed under the influence of light during storage, mediated by bottle hue and thus light transmittance.

Research trials to study these processes will lead to more informed decisions on remedial options and control of this unique and elusive class of green compounds.

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Chapter 2

Effect of closure and packaging type on 3-alkyl-2-methoxypyrazines and other impact odorants of Riesling and Cabernet Franc wine

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Introduction

IBMP, SBMP and IPMP are 3 grape-derived volatile compounds that elicit green and vegetative percepts in wine. Although these MPs can positively influence wine quality in some varieties (1), at higher concentrations they are dominant and unpleasant (2), can mask "fruity/floral" aromas (3), and are associated with wines from cooler climates (4-6) and under-ripe, low quality fruit (5, 6). Recently, lady beetles were identified as a second source of elevated MPs in wine that has been named "ladybug taint" (LBT; (7)). LBT is a wine defect resulting from the undesired incorporation of lady beetles (Coleoptera: Coccinellidae), particularly *Harmonia axyridis* (commonly called the Multicolored Asian Lady Beetle, MALB), into the fermentation process responsible for millions of dollars of lost revenue from downgraded or discarded wine in Southern Ontario and parts of the USA (8). The prevalence of *Harmonia axyridis* in other wine regions, including Italy, France, Spain, South Africa and Argentina (9), suggests that LBT could be or become a more widespread problem for the wine industry.

Regardless of source, MPs can be identified and measured in wines in trace amounts and due to their extremely low sensory detection thresholds – in the high pg/L to low ng/L range (4, 10, 11) - have the potential to significantly impact wine quality. Efforts to reduce MP levels have included both viticultural (12, 13) and enological (14) interventions. However, attempts to decrease MP concentrations in wine using conventional treatments, such as fining, have had limited success (15), and novel approaches are required. Closure and packaging options may offer one such approach.

The capacity for packaging to directly remove volatile compounds through sorptive processes is termed flavor scavenging, and has been well established in the food science/technology literature and exploited commercially. It has been noted particularly with polymer packaging and non-polar flavor compounds (16). While investigating the capacity of natural and agglomerate corks to contribute the taint compound 2,4,6-trichloroanisole to wine, it was observed that these closures had an even greater ability to absorb the compounds (17). Flavor scavenging has since been characterized in wine from a comprehensive bottle-aging trial at the Australian Wine Research Institute, which investigated changes in composition in a Semillon wine after volatile compounds from a range of chemical classes were added and the wine closed with natural corks, synthetic and technical corks, and screwcap closures (18). Other smaller-scale research has examined vanillin permeation through both natural and synthetic corks (19) and thiol interactions with screwcap closures and natural corks (20). These studies concluded that, in general, flavor scavenging in wine is relatively fast and most pronounced for nonpolar volatiles with synthetic-type closures, intermediate with natural corks, and does not occur with screwcap closures (18-20). Although data on flavor scavenging from the popular TetraPak® cartons does not appear in the literature, wine cask bladders (aka 'bag-in-box'), which are made of comparable polymer materials, display marked capacity for flavor scavenging of nonpolar compounds (21). Additionally, we are not aware of any literature on flavor scavenging of MPs in juice or wine.

Winemakers have available to them a range of closure and packaging options which could potentially reduce – or contribute to - MP concentrations in wine. Additionally, TetraPak® cartons (Tpk) have not been investigated with respect to flavor scavenging properties, somewhat surprisingly given their widespread use in the wine industry. Therefore, this study examines the effects of a range of commonly used closures and TetraPak® cartons on MP and other key volatile compounds in Riesling and Cabernet Franc wine over 18 months of bottle-aging.

Materials and Methods

Preparation of Wine and Materials

Riesling and Cabernet Franc wine were chosen for this study due to their importance to the Ontario wine industry and because Cabernet Franc has not previously been investigated for flavor scavenging. Filtered, stabilized wine from grapes grown in the Niagara Peninsula, Ontario was acquired from a commercial winery (Vincor, St. Catharines, ON). The basic initial chemical composition (± SD) of the Riesling and Cabernet Franc wines, was, respectively; titratable acidity (g/L): 7.88 ± 0.38, 4.13 ± 0.00; reducing sugars (g/L): 3.86 ± 0.04, 3.01 ± 0.02; ethanol (% v/v): 8.68 ± 0.97, 11.43 ± 0.17; free SO_2 (mg/L): 26.4 ± 0.8, 19.6 ± 2.0, and pH: 2.83 ± 0.04, 3.72 ± 0.00. Measurements were determined after Iland *et al.* (22), except for ethanol, which was measured by gas-chromatography-flame ionization detection per (23). Initial Riesling and Cabernet Franc MP concentrations (ng/L ± SE), respectively, were; IPMP: 7.4 ± 1.1, 14.3 ± 1.4;

SBMP: 9.6 ± 1.0, 4.48 ± 1.2; IBMP: 8.3 ± 1.3, 26.4 ± 1.7. MPs were analyzed as described below in detail. MPs were analyzed as described below in detail.

IPMP, SBMP and IBMP were acquired from Sigma-Aldrich, Oakville, Ontario (97%, 99%, 99% purity, respectively). In order to achieve ecologically relevant concentrations (5, 25) sufficient for quantification over this longitudinal study, 30 ng/L of each MP was added to the base wines. Each MP was diluted with HPLC grade Methanol and subsequently with Milli-RO water and allowed to equilibrate over a 24-hour period, with regular stirring. Equipment and glassware were thoroughly cleaned with Methanol prior to use to avoid contamination. Deuterated analogs of the MPs ([$^2H_3$]-IPMP, [$^2H_3$]-SBMP, [$^2H_3$]-IBMP) were synthesized for use as internal standards as described in (24).

Four cork-type closures, a roll-on-tamper-evident (ROTE) screwcap, and a Tetrapak® carton were chosen as treatments, because they are in common use in the wine industry and represent a range of material types (***Table 2-1***). Additional SO_2 was added (5 mg/L to Riesling and 20 mg/L to Cabernet Franc) as potassium metabisulfite, to prevent oxidative and microbial spoilage. Wines were filled into 750mL glass Bordeaux bottles (Vineco St. Catharines, Ontario) and closed using standard commercial practices. Wines for finishing in Tpk were filled manually. All bottled and Tpk-packaged wines were stored in a wine cellar (14-16°C) until required for analysis.

Analysis

Duplicate samples (bottles/cartons) were retrieved from the cellar 3, 6, 12 and 18 months after bottling. Samples were poured into 125 mL Nalgene® HDPE bottles (Sigma-Aldrich, Oakville, ON) under nitrogen gas, and sample bottles were tightly closed and covered with laboratory film (Parafilm "M", Pechiney Plastic Packaging, IL, USA) and immediately frozen (-14°C) for later analysis.

3-alkyl-2-methoxypyrazines

MPs were determined from thawed samples taken at bottling, 3, 6, 12 and 18 months using a stable isotope dilution method that uses headspace-solid-phase-microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) as detailed in (24) and summarized below.

Sample preparation & extraction

Samples were prepared with a mixture of isotopically-labelled internal standards ($[^2H_3]$-IPMP, $[^2H_3]$-SBMP and $[^2H_3]$-IBMP in methanol) to achieve 40ng/L of each internal standard, basified with NaOH, to increase the pH to approximately 6.6, and Milli-RO water for a 2.5-fold dilution. Two 10 mL portions of this solution were poured into glass cylinders that contained approximately 30%w/v sodium chloride (Caledon, Hamilton, Ontario) to improve phase transfer, with a small stir bar, and were sealed with a rubber septum for preservation. The sample was then extracted for 30 mins with stirring (1100 rpm) at 40°C on a HS-SPME fiber (StableFlex© Divinylbenzene/Carboxen/PDMS; Supelco, Oakville, Ontario) inserted through the septum into the headspace of the vial. After extraction, the fiber was carefully retracted and inserted into the GC-MS (Agilent 6890GC/5975B with an HP-5MS 5% Phenyl Methyl Siloxane column (30 m, 0.25 mm i.d., 0.25 μm film thickness), Agilent, Oakville Ontario) inlet for sample desorption and analysis. The GC-MS program was as follows: in splitless mode, the injector held with no purge at 250 °C for 5 mins for sample desorption then purged at 50 mL/min for 5 mins to clean the fiber. The oven remained at 40°C for 5 mins, ramped at 3 °C/min up to 110°C, held for 1 min, and ramped at 25 °C/min up to 230 °C. Helium was used as the carrier gas at constant pressure (10.36 psi) with a nominal initial flow (1.2 mL/min). The MSD interface was held at 250 °C while the temperature of the ion source was 200 °C.

Identification and quantification

Identification was achieved using select ion monitoring. For IPMP and [$^2H_3$]-IPMP, respectively, selected mass channels were m/z = 137, 152 and m/z = 140, 155. Ions 137 and 140 were used for quantification, while ions 152 and 155 were used as qualifier ions. For SBMP and [$^2H_3$]-SBMP, respectively, selected mass channels were m/z = 138, 124 and m/z = 141, 127. Ions 124 and 127 were used for quantification, while ions 138 and 141 were used as qualifier ions. For IBMP and [$^2H_3$]-IBMP, respectively, selected mass channels were m/z = 109, 124 and m/z = 112, 127. Ions 124 and 127 were used for quantification, while ions 109 and 112 were used as qualifier ions. All samples were analyzed in duplicate. Area ratios (area of a MP peak/area of corresponding [$^2H_3$]-MP) were calculated from chromatograms and correlated to concentration, based on a standard curve. Three standard curves were developed separately for each MP at varying points over the analysis period. Standards were prepared in a model wine (12.0% v/v ethanol, 4.0 g/L tartaric acid, pH 3.5) and extracted in an identical fashion to wine samples. The first curve was based on six MP concentrations (3, 6, 12, 24, 30, 40 ng/L) and the second two curves on seven (3, 12, 24, 30, 40, 60, 80 ng/L) (see ***Appendix - Figure A-1, Figure A-2).*** The range of R^2 values for the linear regression equations were, for IPMP, SBMP and IBMP respectively, 0.994-0.998, 0.993-0.998 and 0.990-0.998.

Indicator Volatiles

Indicator compounds were chosen to represent the most important classes of wine volatiles based on those previously reported in surveys of a wide range of varietal wines (26, 27). These volatiles will give an indication of the overall sensory changes that may occur in wine and help with any practical recommendations to commercial wine producers. Commercial preparations were obtained (Sigma-Aldrich, Oakville, Ontario) for 5 esters with different alkyl groups (phenethyl acetate, isoamyl acetate; ethyl hexanoate, ethyl caprylate (ethyl octanoate), ethyl caprate (ethyl decanoate)), an alcohol (phenyl ethanol) and a volatile acid (octanoic acid). Indicator volatiles were determined at 3 and 12 months using solid-phase-extraction (modification of (7)) coupled to GC-flame ionization detection using a single chromatographic run (modification of (27)). Three internal standards were selected which are absent in wines, chemically similar to indicator volatiles, and distinct elution times: 3-ethyl-2-hydroxy-valerate (for esters), 3-octanol (for phenyl ethanol) and heptanoic acid (for octanoic acid).

Sample preparation & extraction

Samples were prepared with internal standards (1.90 mg/L 3-ethyl-2-hydroxy-valerate, 32.5 mg/L octanol-3 and 10 mg/L heptanoic acid in HPLC grade methanol) and extracted. The concentrations of internal standards were based on previously reported values for each of the compound classes (26). A C-18, reversed phase column (SupelCLEAN©, Sigma-Aldrich, Oakville, Ontario) was used to extract samples/standards by first conditioning the column (1 mL each of ethyl acetate, 95% v/v methanol, and 10 % v/v methanol), then passing 25 mL of wine sample/standard, drying the column for 10 mins, and finally passing and collecting two 1 mL aliquots of dichloromethane. All samples were concentrated under a nitrogen gas stream to a consistent volume of 0.5 mL. The extract was then injected into the GC-FID (Agilent GC6890 with DB-WAX, 30 m x 0.255 mm x 0.25 μm; J&W Scientific, Oakville, Ontario). The GC-FID oven program was as follows: initially 60°C, ramped 3.0°C/min to 200°C, then ramped 15.0°C/min to 230°C..

Sample quantification

Chromatograms were integrated and the peak height ratios (peak height for target compound/peak height for internal standard) were determined and concentration calculated from calibration curves (see ***Appendix - Figure A-3)***. A 4-point calibration series was used for each compound, ranging from 0.05-0.80 mg/L for the esters, 2.50-120 mg/L for phenyl ethanol and 0.50-12.0 mg/L for octanoic acid. Standards were prepared in a deodorized wine matrix to mimic actual wine composition. Deodorized wines were prepared by adding 1.5g/L activated charcoal (Sigma-Aldrich, Oakville, Ontario) to a white wine (2006 Pinot Grigio, Andrew Peller Ltd., Ontario), stirring for approximately 24 hours, and filtering the solution through a 0.45 μm filter paper. This process was repeated 2-3 times as necessary to remove volatiles, as verified by GC-FID (see ***Appendix - Figure A-5)***, without affecting general wine chemistry parameters, as verified by WineSCAN© analysis (see ***Appendix - Figure A-4***). Average R^2 for the calibration curves were: phenethyl acetate: 0.987; ethyl caprate: 0.984; ethyl caprylate: 0.987; ethyl hexanoate: 0.983; isoamyl acetate: 0.982; phenyl ethanol: 0.947 and octanoic acid: 0.999.

Other Analytes

General wine chemistry parameters were determined at bottling and after 12 months to elucidate potential changes in basic wine chemistry using the methods of (22); pH (by standardized pH meter (AB15 Plus Accumet© Basic, Fisher Scientific, Ontario)), titratable acidity (titrated with 0.1 M NaOH to an 8.2 endpoint), spectrophotometric measures for red and white wines (Genesys 2 spectrophotometer, California) and free and bound SO_2 by the aspiration method. Determinations were performed in duplicate or triplicate.

Reproducibility and variability of analysis

Accuracy and reproducibility of the MP determinations were monitored by quantifying standards of known concentration and by replicate analysis of each wine. After approximately every 15 samples, standards were analyzed to verify methods. The relative standard deviation (RSD) for standards was; IPMP: 5.2%, SBMP: 5.4% and IBMP: 1.8%. Average RSDs from duplicate measurements across all wine samples for all volatile compounds were; IPMP: 7.0%; SBMP: 7.7%; IBMP: 5.7%; phenethyl acetate: 3.7%; ethyl caprate: 1.6%; ethyl caprylate: 2.8%; ethyl hexanoate: 5.5%; isoamyl acetate: 6.0%; phenyl ethanol: 4.6% and octanoic acid: 4.1%. Standard and sample RSDs for MPs are consistent with data from reference (24).

Data treatment

All statistical analyses were performed using XLSTAT-Pro 2008 (Addinsoft, Paris, France). Data were for each analyte for all closure/packaging options at all time points were analyzed using Analysis of CoVariance (ANCOVA) to test for significant variation (see ***Appendix – Table A-6***). The ANCOVA model included analyte concentration as the dependant variable, "time" (in weeks) as the quantitative independent variable, closure/packaging type as the qualitative independent variable, and the interaction between these two factors. When ANCOVA indicated rejection of the null hypothesis ($p(F)<0.05$), one-way Analysis of Variance (ANOVA) tests were completed to investigate effects between closures/packages at specific time points and also between times for specific closure types. If ANOVA supported rejection of the null hypothesis ($p(F)<0.05$), Fisher's Least Significant Difference $(LSD)_{0.05}$ was then used as the means separation test. Principal Components Analysis (PCA) and Correlation Analysis (R values) were conducted on all data at 12 months.

Results and Discussion

3-alkyl-2-methoxypyrazines

MPs were quantified in wines at bottling, and after 3, 6, 12 and 18 months (***Figure 2-1, Figure 2-2***).

3-isopropyl-2-methoxypyrazine

IPMP is associated with perceptions such as "green pea" and "earthy" (28), is the second most prevalent MP present naturally in grapes, and is the causal compound in LBT (7). IPMP concentrations tended to be lower after 12 and 18 months in Cabernet Franc for all closures, but a similar pattern was not observed for Riesling during this trial. A trend of sustained decrease from initial concentrations was observed with Tpk, where values were 23% and 41% lower in Riesling and Cabernet Franc wine, respectively. On occasion, particularly with Agl and Scap after 6 months, IPMP concentration was higher than the initial value, suggesting a contribution from the closure. or interference from a wine component as yet unidentified.

A closure's ability to contribute volatile compounds to wine has been established for trichloroanisoles (29) and also for 2-methoxy-3,5-dimethylpyrazine, an MP constituent of fungally-infected corks (30). However, to our knowledge, IPMP, SBMP and IBMP have not been investigated in this regard, although Allen *et al.* (2) suggest post-bottling contamination as one source of IPMP in wine. Pickering *et al.* (7) reported an average 39% decrease in IPMP in white and red wines affected by *Harmonia axyridis* over 10 months aging in wines closed with moulded synthetic corks. Here, we observe a 10% and 21% decrease after 18 months from concentrations at bottling for white and red wine, respectively, closed with Syn-M. The discrepancy may be related to the different brands of synthetic corks used in the 2 studies, and/or the less accurate method (solid-phase extraction without use of deuterated internal standards) used in the former study.

3-*sec*butyl-2-methoxypyrazine

SBMP is the least studied of MPs found in wine. Naturally present in grapes at lower concentrations than other MPs, SBMP may still play a role in wine aroma, due to an enhancement effect or other sensory interactions (31). Closure/packaging treatments affected SBMP concentration in a similar way in both wines over time. Concentrations of SBMP were lower than initial levels in Tpk wines (average decrease of 27%). Values between 3 and 12 months were most stable for NatC (Riesling) and Scap (Cabernet Franc). However, and most notably, SBMP concentrations increased at some time points for most closures, again suggesting contribution from the closure itself.

3-isobutyl-2-methoxpyrazine

3-Isobutyl-2-methoxypyrazine (IBMP), the most prevalent MP present naturally in grapes, is associated with "bell pepper" aroma (32) and is the most studied in the wine literature. Concentrations in both Cabernet Franc and Riesling responded similarly to treatments. Overall, IBMP decreased significantly in all treatments (***Figure 2-1, Figure 2-2***). After 18 months, the greatest decrease was observed in Tpk and Syn-M and the smallest change was in NatC (Riesling) and Scap (Cabernet Franc). A marked or sustained increase is not observed for any closure/packaging options. Endogenous IBMP concentration has previously been reported as stable during wine storage (18, 33). It is possible that our method has allowed for a more accurate assessment of IBMP changes during storage, and/or the elevated concentrations in our wines have negated potential sensitivity issues in prior studies where levels are closer to the limits of quantitation.

Overall, all 3 MPs were affected by closure and packaging treatments to some extent. After 18 months, the greatest decrease is consistently observed in Tpk, followed by Syn-M and the highest final concentrations observed in Scap and/or NatC (Riesling only; (***Figure 2-1, Figure 2-2***). Detection thresholds in red wine for IBMP and IPMP are 3-10ng/L (4, 11) and 1-2ng/L, respectively (10, 34) and can be even lower for white wine (10). Detection thresholds for SBMP are estimated to be similar to other MPs (13). This high sensitivity of humans to MPs suggests that the concentration differences observed here between some treatments may be perceptible. Sensory analysis is required to confirm this speculation, and determination of difference thresholds for MPs in wine would be of value.

Interestingly, MP concentrations in Tpk decreased between 3-6 months after bottling, after which they remained stable for both wines and all MP species. The Tpk material in contact with wine (polyethylene) is known to remove flavor compounds through FS (16). Surprisingly, given its ubiquitous use, no peer-reviewed literature exists on the influence of Tpk or other multilayer aseptic cartons on wine volatile composition. In wines closed with NatC, all MPs (Cabernet Franc) or IBMP (Riesling) were stable between 3 and 6 months, decreased by approximately 20% and then were stable again. This trend may be associated with the ingress of Oxygen into wine. Lopes *et al.* (35) have reported that oxygen ingress occurs during the first 12 months of storage from within natural corks, after which only trace amounts migrate from the atmosphere to interact with the wine. By contrast, synthetic closures are permeable to atmospheric oxygen after the first month of bottling, while screwcap is essentially impermeable to atmospheric oxygen (35).

Indicator Volatiles

Indicator volatiles, selected to represent the main chemical classes of common wine odorants, were quantified in wines after 3 and 12 months (***Table 2-2***, ***Table 2-3***). This was essential to gain a more comprehensive and encompassing understanding of chemical (and hence sensory) changes that may occur over time in the various packaging options. As expected, Riesling and Cabernet Franc wine had different initial concentrations of these constituents. Three main mechanisms expected to influence wine volatiles during storage are those that occur within the wine matrix, those that occur due to gas permeation, and those due to direct contact with closure/packaging type. Changes that occurred within the wine matrix, regardless of external factors, are distinguished by their prevalence across all treatments. For all closure/packaging types, acetate esters decreased the most, ethyl esters and octanoic acid either increased or decreased slightly and phenyl ethanol remained relatively stable. Over time, changes can occur due to esterification and hydrolysis processes as wine re-establish equilibrium between the esters, alcohols and acids present immediately after fermentation (36). In general, acetate esters decrease and are affected to a greater extent than ethyl esters, which increase over time as the concentration of the corresponding fatty acid decreases slightly, and higher alcohols are generally stable during wine aging (37). These general trends were observed in this trial.

Relating the gas permeability of closures and packages from the literature and the indirect measures included in the present study to changes in volatile concentrations may discriminate effects related to oxygen ingress. TetraPak® cartons appear to have allowed a greater ingress of oxygen into the wines than the bottles, as evidenced by spectrophotometric ($A420_{nm}$)

measurements and changes in free & total SO_2 (38, 39) (***Figure 2-3, Figure 2-4***). This suggestion agrees with earlier findings (38). Tpk wines had the lowest concentration of acetate esters (in Riesling wine) and the highest concentration of ethyl esters after 12 months, but showed no clear trend for other volatiles. Synthetic closures allow for increased oxygen ingress compared to natural cork (39, 40, 41) and subsequent decrease in fruit aroma intensity, likely due to direct oxidative damage to flavour compounds or indirect masking by the formation of aldehydes (39). In the present study, we did not observe a consistent trend of lower concentrations of fruity esters in the synthetic closure treatments, perhaps due to the relatively low inherent concentration of some volatiles and/or the relatively short term of the trial.

Closures and packaging can also affect wine volatiles through direct contact through sorption or migration processes. The relative decrease after 3 months in all volatiles, except phenyl ethanol, in the Agl, Syn-M and Tpk (Cabernet Franc) treatments suggests some sorptive capacity compared to other closures. After 12 months, Agl (Cabernet Franc) and Syn-Ex (Riesling) show the lowest concentrations of ethyl hexanoate, ethyl caprylate (Riesling) isoamyl acetate, phenyl ethanol (Cabernet Franc) and octanoic acid. Interestingly, no increase was observed for any of these volatile compounds, suggesting that migration from closures does not occurr. Godden *et al.* (2001) concluded that closure type affects ester concentration such that ethyl esters are all partially absorbed as a function of increasing alkyl chain length, while small chain esters are unaffected. They also showed that absorption of volatiles varied with closure type, with synthetic corks adsorpting more than natural cork, and no absorption observed with screwcap closures (39). Additionally, Skurray *et al.* (2000) showed that nonpolar volatiles, such as vanillin, can permeate synthetic cork to a greater extent than natural cork (19). The results of the present study agree with this literature; synthetic corks (Syn-M after 3 months and Syn-Ex after 12 months storage) have an increased capacity for volatile sorption compared to natural corks and screwcaps, although the natural cork-based Agl closure also displayed potential flavour scavenging capacity. Overall, the sorption trends were not as clear as some earlier studies, perhaps due to different wine matrices, lower starting concentrations of the selected volatiles, and/or a shorter aging period.

Odour quality and detection thresholds (μg/L) for these compounds indicate the relevance of changes observed, and are as follows: phenethyl acetate: rose/honey/spice, 250; ethyl caprate: fruity/grape, 200; ethyl caprylate: fruity, 5; ethyl hexanoate: apple peel/fruity, 14; isoamyl acetate: banana, 30; phenyl ethanol: rose/honey/spice, 14000; octanoic acid: cheesy/acid, 500 (26, 28, 42). All compounds except phenethyl acetate and ethyl caprate were present at supra-threshold intensity in both wines, indicating that they are likely to influence wine sensory characteristics either directly, or indirectly in conjunction with other volatiles (i.e. MPs). Further investigations using descriptive analysis techniques may be useful to define the sensory impact of these closure/packaging options.

Other Analytes

Other analytes were quantified after 12 months, and included spectrophotometric measures of wine colour, phenolics and free and bound SO_2. Titratable acidity and pH were also measured and did not vary over time or between treatments (see ***Appendix - Table A-5***).

Wines in Tpk had significantly higher $A420_{nm}$ values - an indication of browning - compared with other options (62% higher in Riesling; 44% in Cabernet Franc). Cabernet Franc wine in Tpk also had significantly higher $A520_{nm}$ values - an indication of red pigments -, wine colour density and degree of red pigmentation (see ***Appendix - Table A-1, Table A-2)***. Of the closures we examined, Agl values for these measures ($A420_{nm}$ & $A520_{nm}$) were highest. Contrasting with these results, Buiatti et al. (1997) found no differences between Tpk and bottle-finished white and red wines for phenolic, $A420_{nm}$ and $A520_{nm}$ measurements after 24 months aging.

SO_2 in the free form has both antimicrobial and antioxidant properties that can greatly impact wine quality (22), especially during storage. Tpk performed poorly for both free and bound SO_2 retention (Figure 2). Scap and NatC (Cabernet Franc) preserved the greatest amount of SO_2 and other treatments were intermediary after 12 months. Previous research on closures found that, in general, free SO_2 loss is greater with synthetic closures, intermediary with cork-type closures, and minimal with screw-capped wines (39, 40). By contrast and in a Riesling wine, screwcap closures and natural corks are reported to preserve SO_2 to a similar extent (20). Our results generally agree with these findings, although Agl performed similarly to the synthetic closures. The loss of antioxidants, such as SO_2, is associated with a closure's oxygen permeability. In previous closure trials, it has been suggested that a minimum of 10 mg/L free SO_2 is critical to protect against development of "oxidized" aroma and other negative quality attributes of white wine (39).

Principal Components and Correlation Analyses:

Principal component and correlation analyses were conducted on all data for both wines after 12 months aging. PCA of the Riesling wine (***Figure 2-5***) produced Factors 1 and 2 which account for 78.5% of the variation. Factor 1 is defined by positive loadings for titratable acidity and ethyl caprate and highly negative loadings for IPMP and SBMP. Factor 2 is heavily loaded with spectral measures $A280_{nm}$ (total phenolics) and $A320_{nm}$ (total hydroxycinnamates) and isoamyl acetate. The closures/packaging options are well separated within the PCA space, with

Tpk well discriminated based on its high $A420_{nm}$ values and low free and bound SO_2. The closures are separated along Factor 2, with Scap and Syn-Ex at either end of the axes. Factors 3 and 4 help to further discriminate closure types. Scap is separated from other treatments based on high concentrations for some volatile constituents, while NatC is separated from other closures based on low titratable acidity and high SBMP eigenscores.

The first 2 factors from the PCA of Cabernet Franc (***Figure 2-6***) account for 74.5% of the variation. Factor 1 can be partly interpreted as an index of oxidation, with free and bound SO_2 loading negatively, and color density, red pigmentation, $A420_{nm}$ and $A520_{nm}$ positively loaded. Factor 2 contrasts octanoic acid concentration with wine hue. Tpk is clearly separated from other treatments along Factor 1. Agl is discriminated from other treatments based on lower values for many of the volatile compounds. Scap closed wine is well separated from other closures primarily on the basis of its negative association with wine hue. Factors 3 and 4 discriminate between NatC and Syn-Ex primarily on the basis of small differences in titratable acidity values.

Overall, Tpk and Scap were well-separated from other treatments by PCA, perhaps because they are the most physically different from other closures/packages. Scap was discriminated by its positive association with volatile concentration, consistent with other studies that have reported on its efficacy at preserving wine volatile constituents during storage (20, 39). Tpk correlated with $A420_{nm}$ values and was inversely related to free and bound SO_2, phenomena consistent with the increased gas permeation that has previously been observed in Tetrapak cartons (38). As shown by the narrow angles of their respective eigenvectors (top plots, ***Figure 2-5, Figure 2-6***), MPs were positively correlated to one another. Interestingly, MPs are positively correlated with both free and bound SO_2 in both wines (typical R values range between 0.69 – 0.94), which may imply a role for oxygen ingress in MP loss. Ester concentrations were positively correlated with octanoic acid and each other, except for phenethyl acetate, which was negatively correlated to other volatile compounds in Riesling. As expected, spectrophotometric measures of phenolics were positively correlated in Riesling, and wine color measures positively correlated in Cabernet Franc.

Other Considerations

Overall, the performance of Tpk differentiated it most from the other options, with most differences related to known correlates of elevated oxygen ingress (38-40). The only other study that we are aware of on Tpk concluded that these multilayer aseptic cartons contribute more oxygen to wines than glass (over a 2 yr aging trial), although no significant differences were noted for spectrophotometric measures of phenolics, or $A420_{nm}$ and $A520_{nm}$ values (38). The present data suggest that Tpk has significantly greater levels of oxygen ingress after 12 months; a timeline consistent with manufacturer information (Jim Dolson, Lanpak©, Canada, pers. comm.) and Italian wine industry legislation (38). Tpk wines also had consistently lower concentrations of MPs from 3 months post-packaging, which may be related to flavour scavenging processes, and/or a higher level of gas permeability. While this may suggest Tpk as a viable option for aiding in the remediation of wines with elevated MP levels post-packaging, sensory evaluation is required to fully characterize the effects, given that other differences in Tpk wines (e,g, browning and lower SO_2) are generally regarded as negative quality indicators. In future trials of this nature, direct monitoring of dissolved oxygen concentration in the wines would be beneficial.

Conclusion

This research hypothesized that closure and packaging type will affect 3-alkyl-2-methoxypyrazine (MP) concentration, and was conducted as a longitudinal trial in Riesling and Cabernet Franc wines enriched with MPs. All three MPs were affected by closure/packaging type to some extent, with IBMP the most responsive. MP concentration in wines packaged in Tpk decreased the most, followed by the moulded synthetic cork closure and screwcap and natural cork closures retained the highest levels. Wine concentrations of IPMP and SBMP increased in some treatments after 3 and 6 months, indicating the capacity for some closures to contribute MPs to wine. Some of the indicator volatiles, chosen to represent major chemical classes of odor-active wine compounds, declined with aging, independent of closure/packaging type. Acetate esters showed the greatest decrease, while ethyl esters and phenyl ethanol were generally stable. Agglomerate cork, synthetic corks and TetraPak® all showed some potential sorptive capacity for esters, higher alcohols and volatile acids. TetraPak® was distinct from other treatments after 12 months for many basic wine physico-chemical parameters, including SO_2 and $A420_{nm}$ and we speculate that greater ingress of oxygen accounts for this. Screwcap and natural cork generally preserved higher concentrations of free SO_2. The present study showed the capacity for closure/packaging options to mediate MP concentrations in wine, and generally confirmed previous findings for other volatile species important to wine quality. Further study is required to elucidate changes due to direct contact with packaging material and those related to differential gas permeability, and should include collection of sensory data.

Acknowledgements: Vincor, Ontario, are sincerely thanked for donation of wine for this project. Financial Assistance was provided by the Natural Sciences and Engineering Research Council of Canada, the Wine Council of Ontario and the Grape Growers of Ontario. Thank you to Henry of Pelham Family Estate Winery, Ontario, and Melissa Drouin, Lynda Van Zuiden, Kevin Ker and Dr Tomas Hudlicky, Brock University, for invaluable technical assistance.

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Tables

Table 2-1: Closure/packaging types, material, abbreviation, brand and supplier

Closure/ Packaging	Material/Description	Abbreviation	Brand & supplier
Natural Cork	natural cork, medium quality	**NatC**	Sterisun UFB Natural cork, Scott Laboratories Ltd., ON, Canada
Agglomerate Cork	small natural cork pieces glued together at high pressure	**Agl**	Scott Laboratories Ltd., ON, Canada
Synthetic Cork – Extruded	internal thermoplastic elastomer foam and stiff outer polymer layer on sides, covered by food-grade Silicone coating	**Syn-Ex**	Nomacorc Classic+©, Funk Winemaking Supplies, ON, Canada
Synthetic Cork – Moulded	polyethylene foam, covered by food-grade Silicone coating	**Syn-M**	Supremecorq 45©, Malivoire Wine Company, ON, Canada
Screw Cap	aluminum covered, roll-on tamper evident, teflon liner	**Scap**	Stelvin®, Henry of Pelham Family Estate Winery, ON, Canada
TetraPak® Prisma Aseptic Carton	multi-layer carton (layers from inner to outer: polyethylene (PE), PE, aluminum foil, PE, paper, PE)	**Tpk**	Lanpak® Ltd., Andrew Peller Ltd., ON, Canada

Table 2-2: Indicator volatile concentration in Riesling (mg/L) after bottle aging. Data represent mean values of duplicate measurements of duplicate bottles ±SEM. Means sharing the same letter do not differ significantly at specific time points (Fisher's Protected $LSD_{0.05}$).

Analyte	TIME	Natural cork	Agglomerate cork	Synthetic cork - extruded	Synthetic cork - moulded	Screwcap	TetraPak
Phenethyl acetate	bottling			0.213 ±0.000			
	3 mos	0.145b ±0.003	0.168a ±0.008	0.150b ±0.001	0.154b ±0.006	0.154b ±0.002	0.145b ±0.004
	12 mos	0.141a ±0.001	0.141a ±0.000	0.140a ±0.002	0.130a ±0.006	0.130a ±0.006	0.118b ±0.001
Ethyl caprate	bottling			0.141 ±0.002			
	3 mos	0.137a ±0.003	0.106b ±0.003	0.139a ±0.001	0.142a ±0.005	0.139a ±0.002	0.138a ±0.004
	12 mos	0.111b ±0.001	0.108b ±0.002	0.112b ±0.003	0.121b ±0.007	0.123b ±0.010	0.140a ±0.001
Ethyl caprylate	bottling			0.207 ±0.011			
	3 mos	0.236a ±0.008	0.127bc ±0.015	0.198ab ±0.015	0.155bc ±0.039	0.089c ±0.000	0.167ab ±0.046
	12 mos	0.108b ±0.009	0.120b ±0.006	0.101b ±0.006	0.120b ±0.018	0.202a ±0.050	0.227a ±0.014
Ethyl hexanoate	bottling			0.214 ±0.005			
	3 mos	0.194a ±0.014	0.163a ±0.031	0.172a ±0.014	0.175a ±0.013	0.194a ±0.006	0.197a ±0.007
	12 mos	0.152a ±0.002	0.170a ±0.006	0.128a ±0.009	0.176a ±0.014	0.182a ±0.017	0.188a ±0.008
Isoamyl acetate	bottling			0.571 ±0.008			
	3 mos	0.338a ±0.021	0.252a ±0.052	0.323a ±0.019	0.351a ±0.033	0.376a ±0.007	0.325a ±0.018
	12 mos	0.110b ±0.001	0.130a ±0.009	0.103b ±0.002	0.138a ±0.006	0.133a ±0.003	0.110b ±0.005
Phenyl ethanol	bottling			39.121 ±0.461			
	3 mos	36.812a ±1.464	39.447a ±4.348	36.823a ±1.316	38.083a ±1.005	38.655a ±0.495	39.669a ±1.227
	12 mos	34.549a ±1.408	37.506a ±1.667	35.232a ±1.148	40.548a ±1.080	43.425a ±2.195	42.436a ±1.848
Octanoic acid	bottling			6.139 ±0.204			
	3 mos	5.243a ±0.124	3.480b ±0.315	5.063a ±0.168	4.856a ±0.327	5.031a ±0.225	5.309a ±0.111
	12 mos	3.966cd ±0.174	4.187c ±0.264	3.298d ±0.025	4.414bc ±0.173	5.444a ±0.492	5.127ab ±0.189

Table 2-3: Indicator volatile concentration in Cabernet Franc (mg/L) after bottle aging. Data represent mean values of duplicate measurements of duplicate bottles ±SEM. Means sharing the same letter do not differ significantly at specific time points (Fisher's Protected $LSD_{0.05}$).

Analyte	TIME	Natural cork	Agglomerate cork	Synthetic cork - extruded	Synthetic cork - moulded	Screwcap	TetraPak
Phenethyl acetate	bottling			**1.021** ±0.044			
	3 mos	**0.855ab** ±0.040	**0.855ab** ±0.066	**0.906a** ±0.016	**0.696c** ±0.026	**0.890a** ±0.031	**0.742bc** ±0.064
	12 mos	**0.371b** ±0.024	**0.353b** ±0.009	**0.361b** ±0.020	**0.371b** ±0.017	**0.400ab** ±0.009	**0.469a** ±0.050
Ethyl caprate	bottling			**0.105** ±0.014			
	3 mos	**0.128a** ±0.001	**0.121a** ±0.002	**0.124a** ±0.001	**0.121a** ±0.001	**0.127a** ±0.001	**0.108b** ±0.007
	12 mos	**0.090bc** ±0.003	**0.095b** ±0.000	**0.090bc** ±0.003	**0.085c** ±0.000	**0.091bc** ±0.004	**0.104a** ±0.000
Ethyl caprylate	bottling			**0.122** ±0.024			
	3 mos	**0.169bc** ±0.046	**0.089d** ±0.000	**0.145cd** ±0.033	**0.223ab** ±0.006	**0.249a** ±0.016	**0.162bcd** ±0.023
	12 mos	**0.097bc** ±0.006	**0.091c** ±0.000	**0.104bc** ±0.007	**0.091c** ±0.000	**0.137ab** ±0.003	**0.148a** ±0.035
Ethyl hexanoate	bottling			**0.233** ±0.059			
	3 mos	**0.190a** ±0.008	**0.162a** ±0.012	**0.176a** ±0.007	**0.166a** ±0.008	**0.211a** ±0.007	**0.196a** ±0.008
	12 mos	**0.187a** ±0.022	**0.176a** ±0.005	**0.190a** ±0.032	**0.183a** ±0.014	**0.246a** ±0.013	**0.228a** ±0.015
Isoamyl acetate	bottling			**0.463** ±0.052			
	3 mos	**0.355ab** ±0.018	**0.326bc** ±0.031	**0.345abc** ±0.027	**0.289c** ±0.016	**0.410a** ±0.006	**0.311bc** ±0.026
	12 mos	**0.246a** ±0.043	**0.198a** ±0.007	**0.263a** ±0.050	**0.302a** ±0.022	**0.308a** ±0.035	**0.329a** ±0.024
Phenyl ethanol	bottling			**41.339** ±6.479			
	3 mos	**35.107a** ±0.720	**36.178a** ±1.380	**36.345a** ±0.601	**36.728a** ±1.524	**34.412a** ±0.946	**34.479a** ±1.620
	12 mos	**37.622a** ±3.470	**31.139a** ±0.143	**37.057a** ±4.166	**41.182a** ±2.691	**39.658a** ±3.520	**38.792a** ±2.501
Octanoic acid	bottling			**1.967** ±0.080			
	3 mos	**1.895a** ±0.029	**1.784a** ±0.069	**1.949a** ±0.047	**1.822a** ±0.033	**2.007a** ±0.026	**1.577a** ±0.115
	12 mos	**1.708a** ±0.126	**1.506a** ±0.024	**1.599a** ±0.079	**1.661a** ±0.066	**1.805a** ±0.147	**1.698a** ±0.029

Figures

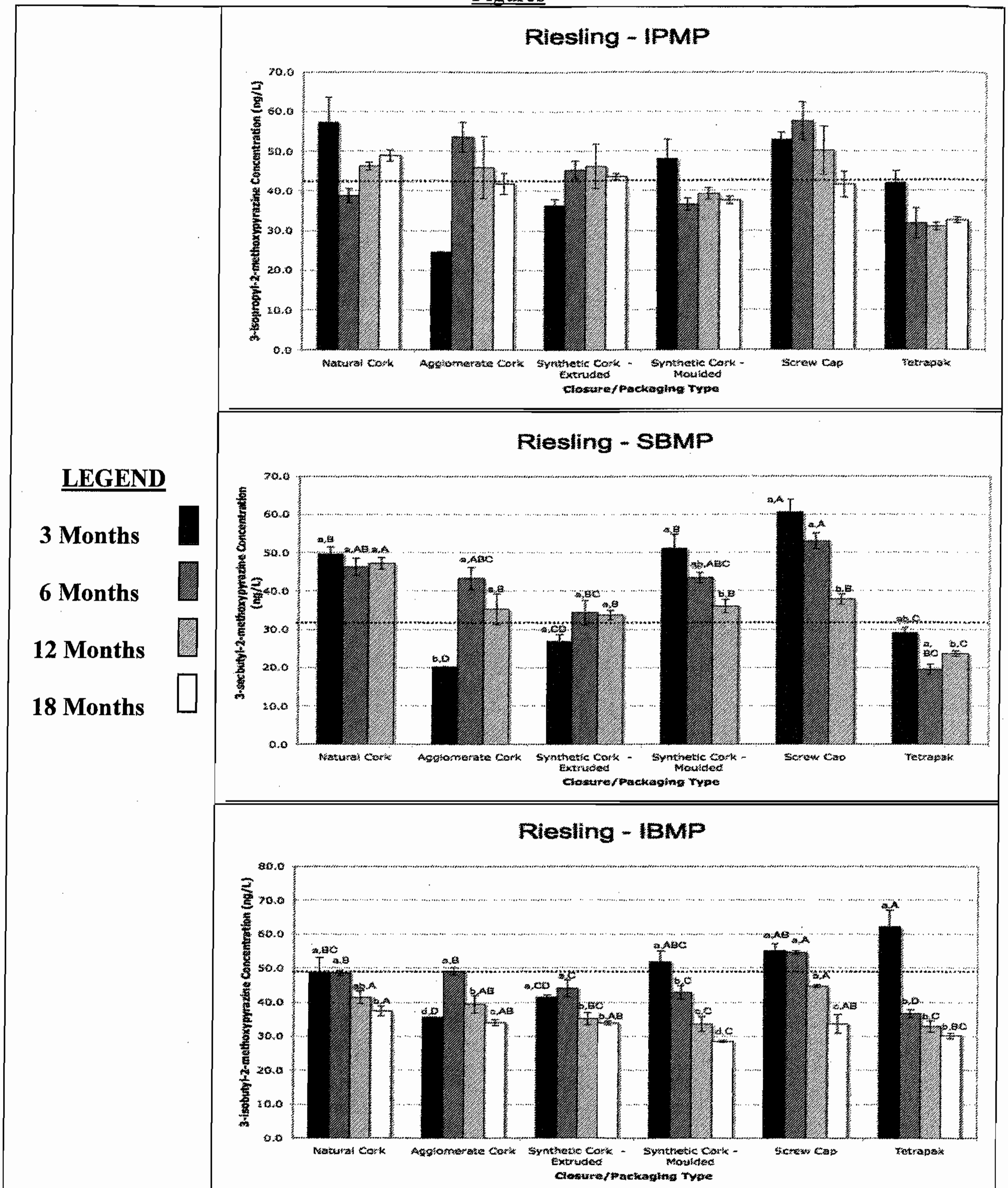


Figure 2-1: Concentration of 3-alkyl-2-methoxypyrazines (MP) in Riesling spiked with 30 ng/L of isopropyl-, *sec*butyl-, and isobutyl-MP. Data represent values of duplicate or triplicate measurements of duplicate bottles ±SEM. Means with letters are those which differ significantly, and of those, means sharing the same letter do not differ in groups across time [lowercase] or at specific time points [uppercase] (Fishers Protected $LSD_{0.05}$). Dashed line indicates initial MP concentration at bottling.

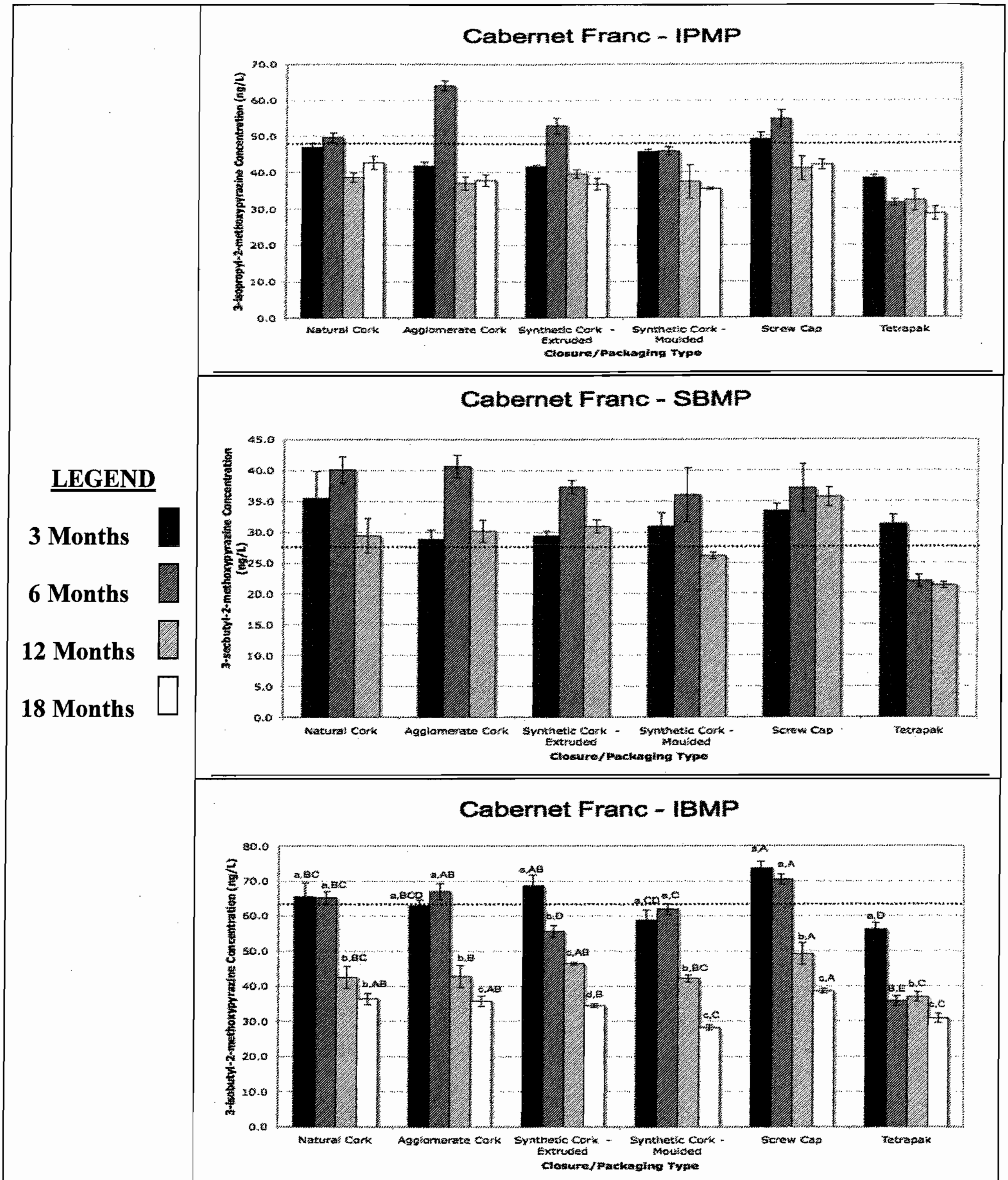


Figure 2-2: Concentration of 3-alkyl-2-methoxypyrazines (MP) in Cabernet Franc spiked with 30 ng/L of isopropyl-, *sec*butyl-, and isobutyl-MP. Data represent values of duplicate or triplicate measurements of duplicate bottles ±SEM. Means with letters are those which differ significantly, and of those, means sharing the same letter do not differ in groups across time [lowercase] or at specific time points [uppercase] (Fishers Protected $LSD_{0.05}$). Dashed line indicates initial MP concentration at bottling.

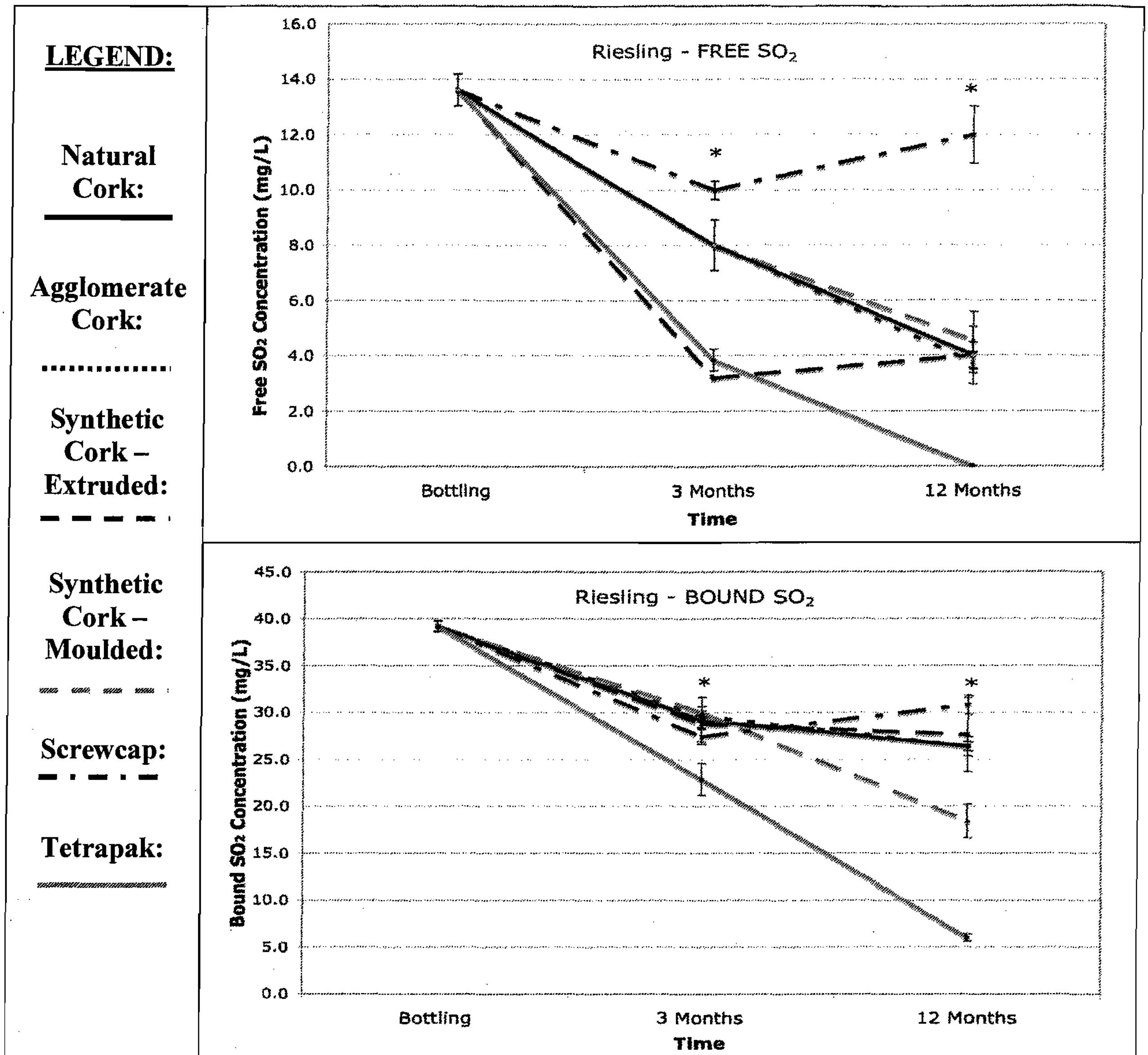


Figure 2-3: Free and Bound Sulfur Dioxide in Riesling wine. Data represent mean values of duplicate measurements of duplicate bottles ±SEM. An asterisk is above time points with significantly different means (Fisher's Protected $LSD_{0.05}$).

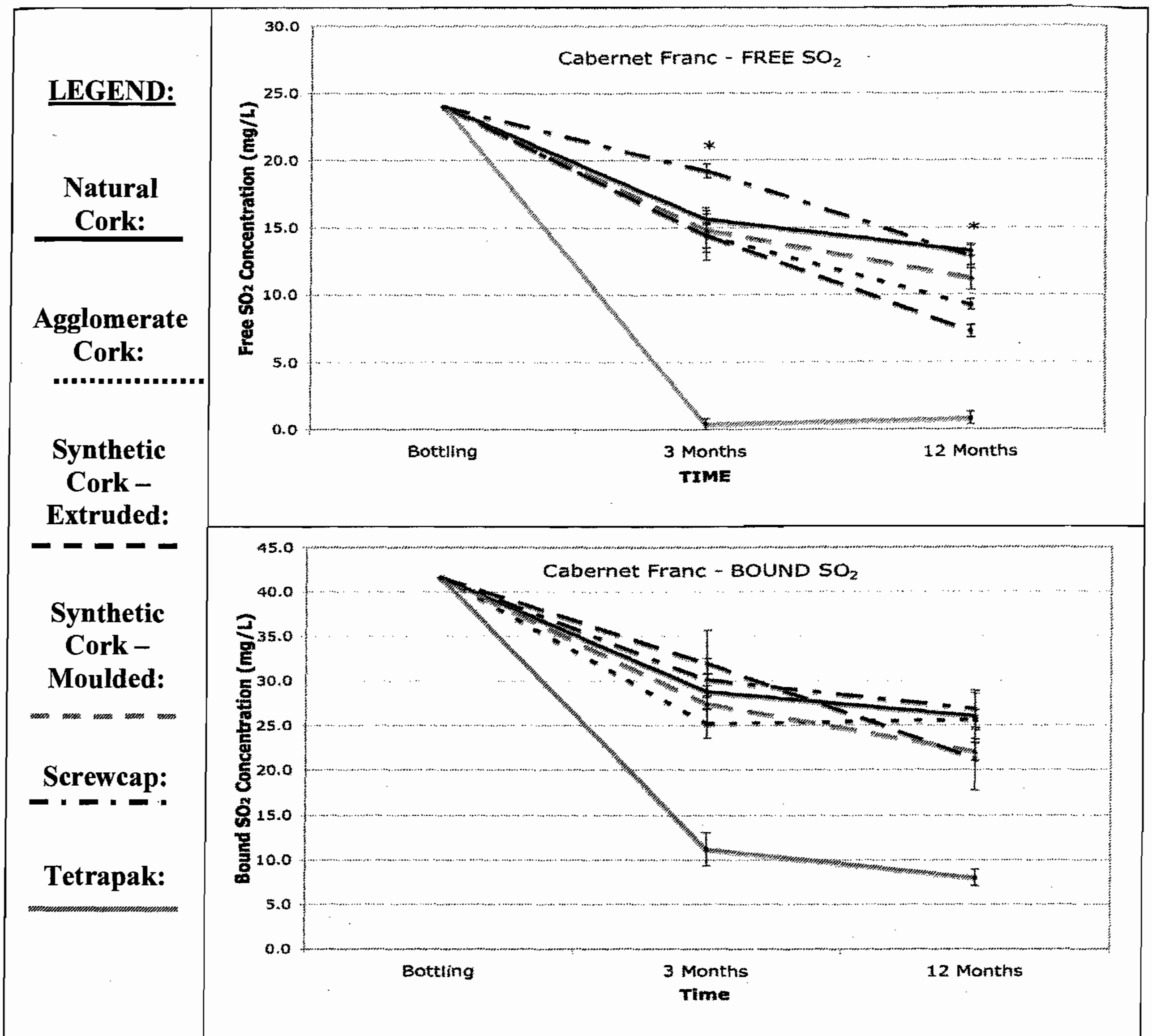
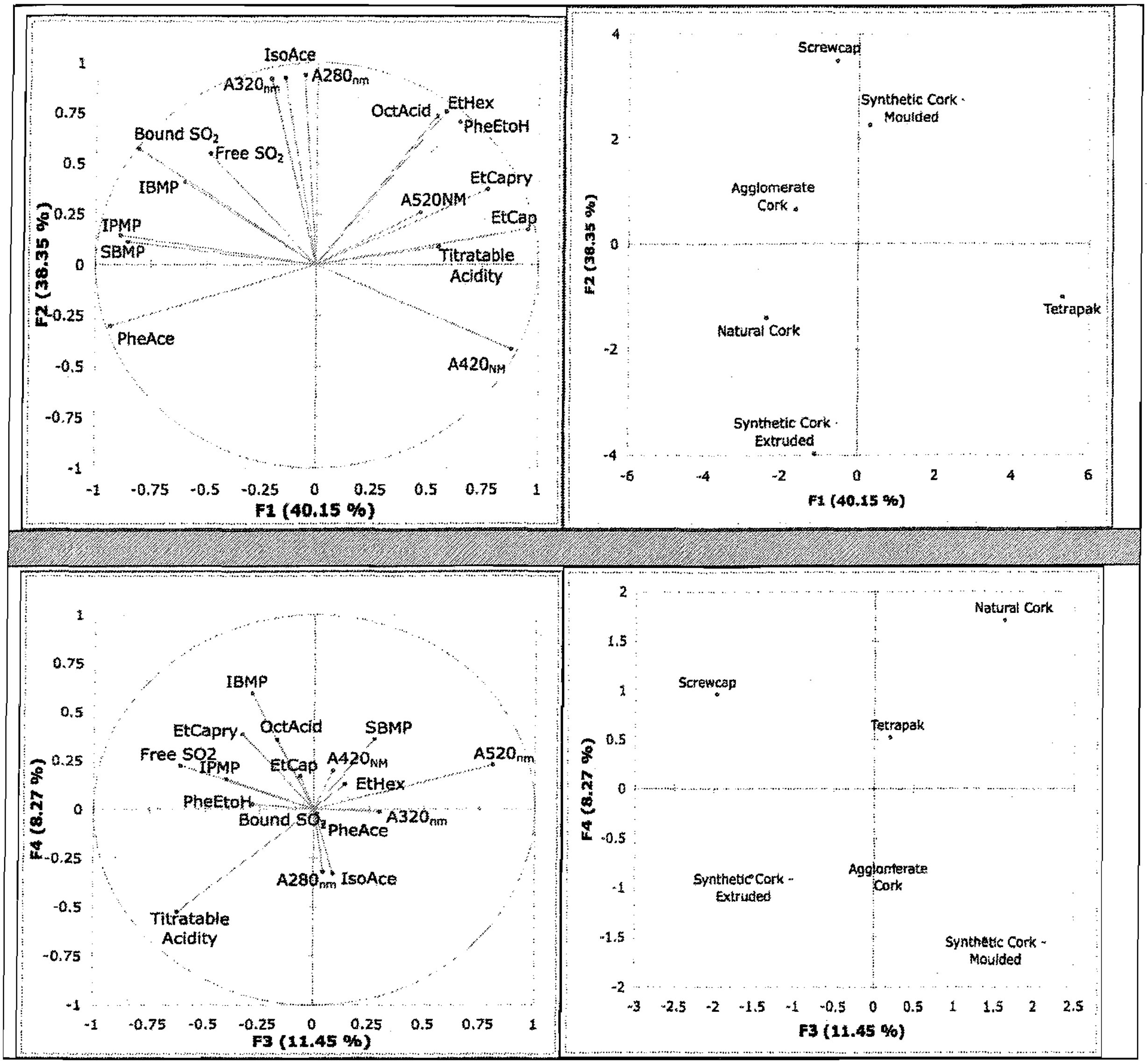
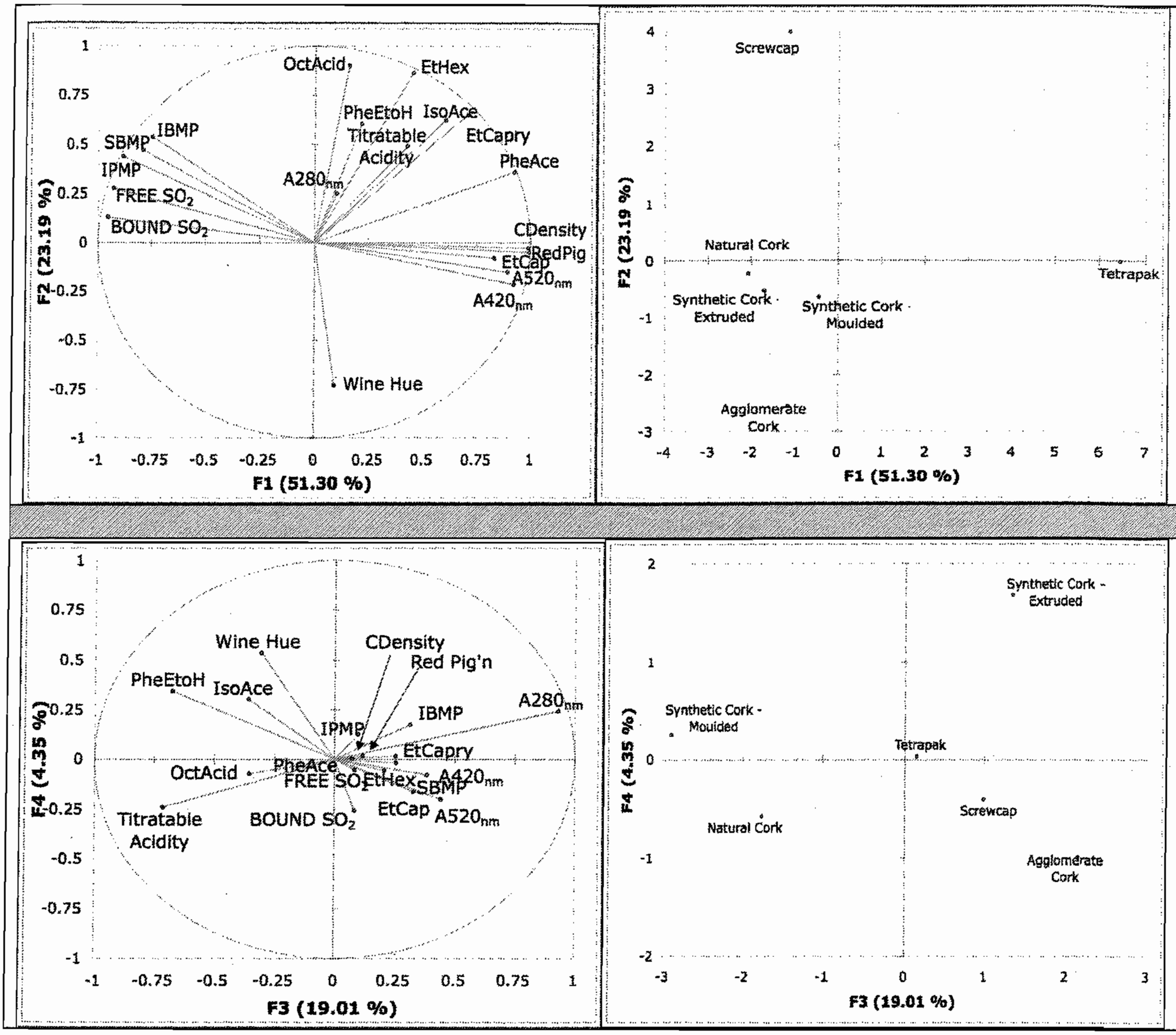


Figure 2-4: Free and Bound Sulfur Dioxide in Cabernet Franc wine. Data represent mean values of duplicate measurements of duplicate bottles ±SEM. An asterisk is above time points with significantly different means (Fisher's Protected $LSD_{0.05}$).



Abbreviations: IPMP: 3-isopropyl-2-methoxypyrazine; SBMP: 3-secbutyl-2-methoxypyrazine; IBMP: 3-isobutyl-2-methoxypyrazine; PheAce: phenethyl acetate; EtCap: ethyl caprate; EtCapry: ethyl caprylate; EtHex: ethyl hexanoate; IsoAce: isoamyl acetate; PheEtoH: phenyl ethanol; OctAcid: octanoic acid.

Figure 2-5: PCA biplot for Riesling wine after 12 months bottle age. Factor 1 vs. Factor 2 (top) and Factor 3 vs. Factor 4 (bottom)



Abbrevations: IPMP: 3-isopropyl-2-methoxypyrazine; SBMP: 3-secbutyl-2-methoxypyrazine; IBMP: 3-isobutyl-2-methoxypyrazine; PheAce: phenethyl acetate; EtCap: ethyl caprate; EtCapry: ethyl caprylate; EtHex: ethyl hexanoate; IsoAce: isoamyl acetate; PheEtoH: phenyl ethanol; OctAcid: octanoic acid; CDensity: wine colour density; RedPig: Degree of Red Pigmentation

Figure 2-6: PCA biplot of Cabernet Franc wine after 12 months bottle age. Factor 1 vs. Factor 2 (top) Factor 3 vs. Factor 4 (bottom)

Chapter 3

Effect of light and temperature on 3-alkyl-2-methoxypyrazine concentration and other impact odorants of Riesling and Cabernet Franc wine during bottle aging

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Introduction

Odor-active compounds are critical determinants of the flavour and overall quality of wine. 3-alkyl-2-methoxypyrazines (MPs) are powerful odorants ubiquitous in the natural world (1), and components of many wines, where thresholds have been reported as low as 320 pg/L (2). 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*butyl-2-methoxypyrazine (SBMP) and 3-isobutyl-2-methoxypyrazine (IBMP) are secondary plant metabolites that are most abundant in grape varieties from the Bordeaux region in France (3-5). These MPs can contribute to the desired varietal character of certain wines (6, 7), but, at higher levels, are responsible for vegetative and herbaceous aromas considered detrimental to wine quality (8, 9). During the latter stages of grape maturation MPs degrade (10), and concentrations at harvest are higher in under-ripe fruit (3, 11-13) and in grapes grown in cooler climates (14, 15). Elevated levels of MPs in wine can also be due to incorporation of the lady beetle *Harmonia axyridis* (Coleoptera: Coccinellidae;

HA) with grapes at harvest (16), where excretion or extraction of IPMP from the beetle (8) produces off-flavors known as ladybug taint (LBT; (17)). *HA* can be found in vineyards in large numbers around the time of commercial grape harvest (18), and are found in many winemaking regions of the world, including Italy, France, Spain, South Africa and Argentina (19).

Pre-fermentation settling of white wines can reduce MP levels (20), however they are relatively stable during fermentation (21, 22), and some commercial yeast strains may even produce MPs during the fermentation (23). They are generally resilient to standard wine fining practices (24), and recent investigations have shown some capacity for post-bottling modification by closures and packaging, likely through migration and sorptive processes (25). Thus, other approaches to remediation of high MP wines have been advocated. Choice of bottle hue when bottling and light and temperature conditions during storage and retail display may be additional control points whereby MP concentrations can be further modified.

Both light exposure and increased storage temperature have previously been shown to affect some wine constituents. In general, wavelengths in the UV spectrum and the blue portion of the visible spectrum (~350nm-500nm) adversely affect wine and other food products (e.g., beer, milk) in the presence of riboflavin, producing "light-struck" or "sunlight" sensory changes (26). In wine, these changes are believed to result from the photo-activation of riboflavin (vitamin B2) causing the formation of volatile sulphur compounds from sulphur-containing amino acid degradation and/or the selective decomposition of esters (26). The most common hues, clear, green and amber hues, transmit 95%, 50%, and 10% of 350nm-550nm light, respectively (27). Temperature may also affect normal bottle-ageing through mediating reaction rates (28). Increased storage temperature produces "quick-ageing" effects associated with advanced oxidation; volatile concentration approach chemical equilibrium (28, 29), yellow pigments increase and in general, phenolic compounds decrease (30).

Light and temperature have also been linked to MPs. Levels of MPs in grapes are significantly lower in warmer climates as a result of increased light exposure (11, 31) and/or temperature (15). Additionally, IBMP and IPMP have been shown to photo-degrade in aqueous solution (32), and IBMP is substantially reduced in red wine subject to thermo-vinification (33). Surprisingly, no research appears in the peer-reviewed literature informing the hypothesis that light, and its mediation by bottle hue or storage temperature can influence MP composition in finished wines. In addition, information on how cellaring conditions might influence other impact odorants in wine is limited. These considerations form the basis of this study.

Materials and Methods

Preparation of wine and materials

Riesling and Cabernet Franc wine from grapes grown in the Niagara Peninsula, Ontario, were used in this study based on their importance to both the local and global wine industries. Basic chemical composition of the base wines was determined using the methods of Iland *et al.* (34), except for ethanol analysis, which was determined using GC-FID after (35). Values ± SD for Riesling and Cabernet Franc wines, respectively, were; titratable acidity (g/L): 7.88 ± 0.38, 4.13 ± 0.00; reducing sugars (g/L): 3.86 ± 0.04, 3.01 ± 0.02; ethanol (% v/v): 8.68 ± 0.91, 11.43 ± 0.16; free SO_2 (mg/L): 26.4 ± 0.8, 19.6 ± 2.0, and pH: 2.83 ± 0.04, 3.72 ± 0.00. Initial Riesling and Cabernet Franc MP concentrations (ng/L ± SE), respectively, were; IPMP: 7.4 ± 1.1, 14.3 ±

1.4; SBMP: 9.6 ± 1.0, 4.4 ± 1.2; IBMP: 8.3 ± 1.3, 26.4 ± 1.7. MPs were analyzed as described below in detail.

IPMP, SBMP and IBMP were acquired from Sigma-Aldrich, Oakville, Ontario (97%, 99%, 99% purity, respectively). In order to achieve ecologically relevant concentrations (4, 13) sufficient for quantification over this longitudinal study, 30 ng/L of each MP was added to the base wines. and then equilibrated over 24 hours with regular stirring. After equilibration, wines were bottled or packaged. A further 5 mg/L (Riesling) or 20 mg/L (Cabernet Franc) of SO_2 (as potassium metabisulfite) was added immediately prior to bottling. Wines were bottled in 750mL glass Bordeaux bottles (Vineco, St. Catharines, Ontario) and closed with Sterisun® natural corks (Scott Laboratories, Ontario) using standard commercial practice. Bottles were placed upright for 7 days before storing horizontally for all conditions to allow time for closures to adjust to the bottle.

Three chambers were prepared for storage of the wines under specific lighting and temperature conditions: Condition 1 ("Light and Ambient Temp"), for examining the influence of clear, green and amber bottle hues at 22 °C; Condition 2 ("Dark and Ambient Temp") and Condition 3 ("Dark and Cellar Temp"), as represented in ***Figure 3-1***. Light exposure for Condition 1 was provided by 15W compact fluorescent light bulbs (*Phillips Marathon*® Energy Saving Mini-Twister) placed ~40cm above the bottles at 1 bulb per 10 bottles. Bottles in Condition 2 were stored in sealed cardboard wine cases at ambient temperature, and wines in Condition 3 were stored in a Uni-THERM© refrigerator (Grand Haven, Michigan), at a constant temperature of 12°C.

Analysis

Sample Preparation

Duplicate bottles were retrieved from chambers for analysis at 3, 6, and 12 months after bottling. 100 mL samples were poured into Nalgene® HDPE bottles (Sigma-Aldrich, Oakville, ON) under nitrogen gas, then bottles were closed, covered with laboratory film (Parafilm "M", Pechiney Plastic Packaging, IL, USA) and promptly frozen for future analysis.

3-alkyl-2-methoxypyrazines and other analytes

MPs were determined from thawed samples taken at bottling, 3, 6, and 12 months using a recently developed stable isotope dilution method that uses headspace-solid-phase-microextraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC-MS) as detailed in (20) and summarized in *Chapter 2*. Indicator volatiles and other analytes were determined as described in *Chapter 2*.

Reproducibility and variability of analysis

Accuracy and reproducibility of the MP determinations were monitored by quantifying standards of known concentration and by replicate analysis of each wine. After approximately every 15 samples, standards were analyzed to verify methods. The relative standard deviation (RSD) for standards was: IPMP: 3.5%; SBMP: 3.7%; IBMP: 3.1%. Average RSDs from duplicate measurements across all wine samples for all volatile compounds were; IPMP: 8.0%; SBMP: 7.1%; IBMP: 8.1%; phenethyl acetate: 3.0%; ethyl caprate: 2.0%; ethyl caprylate: 4.9%; ethyl hexanoate: 4.0%; isoamyl acetate: 3.3%; phenyl ethanol: 5.8%; octanoic acid: 3.6%. Standard and sample RSDs for MPs are consistent with data from reference (20).

Data Treatment

All statistical analyses were performed using XLSTAT-Pro 2008 (Addinsoft, Paris, France). Data for each analyte for all closure/packaging options at all time points were analyzed using Analysis of CoVariance (ANCOVA) to test for significant variation (see ***Appendix – Table A-6)***. The ANCOVA model included analyte concentration as the dependant variable, "time" (in weeks) as the quantitative independent variable, closure/packaging type as the qualitative independent variable, and the interaction between these two factors. When ANCOVA indicated rejection of the null hypothesis ($p(F)<0.05$), one-way Analysis of Variance (ANOVA) tests were completed to investigate effects between closures/packages at specific time points and also between times for specific closure types. If ANOVA supported rejection of the null hypothesis ($p(F)<0.05$), Fisher's Least Significant Difference $(LSD)_{0.05}$ was then used as the means separation test. Principal Components Analysis (PCA) and Correlation Analysis (R values) were conducted on all data at 12 months.

Results and Discussion

3-alkyl-2-methoxypyrazines

MPs were quantified in wines at bottling and after 3, 6, and 12 months (***Figure 3-2, Figure 3-3***). Over 12 months, IPMP concentrations were relatively stable or displayed small decreases, and were not consistently affected by light and/or temperature conditions. However, after 12 months IPMP tended to be higher in light-excluded Riesling wines, and, within light treatments, higher in amber bottles compared with other hues for both Riesling and Cabernet Franc. SBMP concentration was unaffected by light or temperature during storage. In Riesling, an average increase of 8 ng/L above concentration at bottling is observed after 12 months, and an increase in IPMP concentration at 3 months in seen in 2 conditions. IBMP decreased with time in both wine styles regardless of light or temperature condition, which did not consistently affect concentrations.

The pattern of increase in IPMP and SBMP concentration in some wines is consistent with that reported by (25) in wine closed with natural cork, and we speculate due to migration of these MPs from the closure. Migration of a methoxypyrazine from cork into wine has been reported by other researchers (38). Some evidence suggests that IBMP and IPMP photodegrade in ripening grapes (11, 31), although the mechanisms are unclear (31). We found no consistent trend indicative of photodegradation of MPs in wine, consistent with Pickering *et al.* (24) who reported no significant effect of UV or visible light on IPMP when wine was passed through a light reactor. It is possible that any light-mediated degradation of MPs may be obscured by their apparent migration from some natural cork closures into the wine.

Our data suggest that IBMP shows the greatest reduction during bottle-ageing, consistent with Blake *et al.* (25) in their 18 months trial under cellar conditions. While sorption of MPs by cork has previously been noted and may account for some of this loss, it is unlikely sufficient, as alternative closures, including screwcaps, can demonstrate similar decreases during cellaring (25). It is possible that MPs, and particularly IBMP, are becoming incorporated in polyphenolic complexes and precipitating from solution over time. This speculation gains some support from the greater drop in IBMP concentration observed in (phenolic-rich) red wine in both (25) and the current study.

Indicator Volatiles

Indicator volatiles were quantified in wine at bottling and 3 and 12 months post-bottling (***Figure 3-4, Figure 3-5, Figure 3-6***). This was included in the research design in order to understand how other key chemical constituents of wines were affected by storage conditions. Selected indicator volatiles acted as representative compounds for a variety of volatile classes active in wine aroma and overall quality. The predominant changes occurring during bottle ageing involve the transformation of volatile constituents as wines re-establish a chemical equilibrium between acids, alcohols and corresponding esters (39). These reactions have temperature-dependant rates (28).

In the present study, concentrations of acetate esters decreased with storage time, regardless of condition, consistent with known equilibrium processes (40). However of all indicator volatiles monitored, the acetate esters were also the most affected by storage conditions; phenethyl acetate and isoamyl acetate decreased at a greater rate in ambient temperature conditions compared with 12°C. This may be due to enhanced ester hydrolysis, which increases linearly with temperature (41) and has previously been reported to be most pronounced with acetate esters (30). Ethyl esters, particularly ethyl hexanoate, tended to be stable with time and did not vary consistently with storage conditions. This result was expected, as ethyl esters are present in young wine at concentrations close to chemical equilibrium (30) and hydrolyze relatively slowly (41). Interestingly, ethyl caprylate in Riesling after 12 months was significantly higher in the dark + ambient temperature condition. Bottle hue did not influence acetate or ethyl ester composition.

Both phenyl ethanol and octanoic acid concentration are similar for all treatments and remain relatively stable over time. At 3 months light-exposed treatments tend to have higher concentrations of octanoic acid than wines stored under dark conditions, although this effect is not seen after 12 months. Again, bottle hue did not affect the concentration of these analytes.

Overall, these data closely agree with the results from Marais and Pool (28) who looked at temperature effects during storage of a Chenin Blanc wine over 12 months.

Sulphur dioxide

Free and bound SO_2 measurements were taken at bottling and after 3 and 12 months aging. Titratable acidity and pH were also measured and did not vary over time or between treatments (data not shown). Free and bound SO_2 retention tended to be higher in light-excluded conditions, while temperature during storage did not affect SO_2 preservation (***Figure 3-7, Figure 3-8***). Bottle hue also influenced free SO_2 concentration, with retention (averaged across both wine styles) greatest in amber (55.5%), intermediate with green (42.5%), and lowest in clear bottles (33.8%). A similar pattern is apparent for bound SO_2. Chemical reactions in wines, which occur with light exposure, involve the reduction the photosensitizing agent, riboflavin, which in turn becomes an oxidizing agent, catalyzing the dehydration of sulphur-containing amino acids and production of volatile sulphur compounds (42). We speculate that light exposure, and the increased exposure to wine in clear hue bottles increased riboflavin photo-activation and this oxidizer was responsible for the differential decrease in SO_2, a reducing agent that is abundant in wine.

Principal Components and Other Analyses

Principal components analysis and correlation analysis were performed on all data collected after 12 months. Included in this were spectrophotometric measurements of wine colour and phenolics (see ***Appendix - Table A-3, Table A-4)***. Factors 1 and 2 of the PCA analysis of Riesling after 12 months storage account for approx. 70% of the total variation (***Figure 3-9)***. Factor 1 is not closely associated with any particular eigenvector, while Factor 2 is heavily loaded with bound SO_2 and, to a lesser extent, free SO_2. Wine from the Dark + Ambient condition is well separated from other wine largely by its higher values for these parameters. Wines of varying bottle hue are separated by Factors 3 and 4, which together account for approx. 30% of the variation. Separation is largely based on their relative values for $A420_{nm}$, phenyl ethanol, ethyl hexanoate and SO_2. Factors 1 and 2 of the PCA analysis of Cabernet Franc account for approx. 69% of the total variation (***Figure 3-10***). Factor 1 is positively loaded with isoamyl acetate, total red pigments and wine hue, and negatively loaded with $A420_{nm}$ and degree of red pigmentation. Factor 2 is positively loaded with ethyl caprylate and negatively loaded with bound SO_2. Dark + 12°C is well discriminated from other wines, largely due to its significantly higher values for A520nm, total red pigments and colour density (data not shown). Bottle hues are separated by Factors 3 and 4, which account for approx. 31% of the variation. Green is discriminated based on its positive association with ethyl caprylate, and clear bottles are differentiated from those of green and amber hue based on their relatively low scores for A280nm and total phenolics.

Browning has previously been reported to increase with storage temperature in both white and red wines (43, 44), in agreement with the trends in A420nm values observed here. Browning in white wine is inhibited by SO_2 (43), which in this trial was reduced in light-exposed conditions; suggesting that the combination of clear bottles and elevated storage temperature is not optimal for protecting against premature browning and perhaps other negative quality indicators in white wine. While not statistically significant, Cabernet Franc wines stored in clear bottles were 8% and 9% lower for A280nm and total phenolic measures, respectively, compared with wine stored in amber and green bottles. This agrees with the differences (decrease) in phenolic compounds observed in sherry stored in clear vs. topaz bottles (45).

Correlation analysis (data not shown) on Riesling wine produced relatively few associations, while many analytes in Cabernet Franc were positively correlated. Interestingly, octanoic acid is strongly correlated with IBMP in both wines (0.952 in Riesling, 0.986 in Cabernet Franc), an unexpected association. IPMP and SBMP are positively correlated in Cabernet Franc (0.892); however, IBMP in Cabernet Franc and all MPs in Riesling wines, were not. This lack of association is also unexpected, given their similar chemical structure, but supports the earlier result that MPs are not equally affected by storage conditions, including closures, in this trial.

Conclusion

We hypothesized that light exposure and temperature will affect 3-alkyl-2-methoxypyrazines and other volatile constituents in Riesling and Cabernet Franc wines enriched with MPs during a 12-month storage trial. These conditions did not consistently influence MP concentrations, a result which may have been confounded with suspected migration of MPs from the cork closures in some wines. This speculation warrants further investigation. In contrast, both light and temperature affected many of the other volatile and non-volatile constituents examined. The combination of light-exclusion and cooler storage conditions tend to associate with increased retention of acetate esters, free and bound SO_2, phenolic compounds (in red wine), and a lower browning index, all potential indicators of higher wine quality. This finding is consistent with anecdotal information concerning optimal cellaring conditions for wine, but should be further verified with sensory evaluation. This is particularly relevant in the context of MP-rich wines, where changes in other impact odorants during aging may affect the perceived green characters of the wines.

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<u>Figures</u>

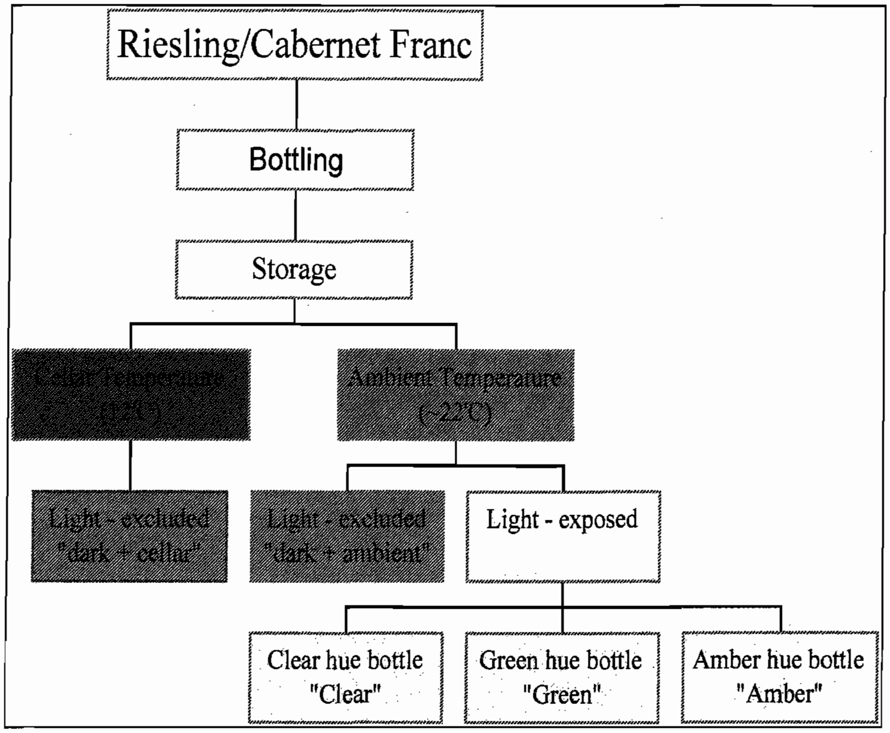


Figure 3-1: Experimental design of light/temperature chambers for wine storage over longitudinal trial (modification of (24)).

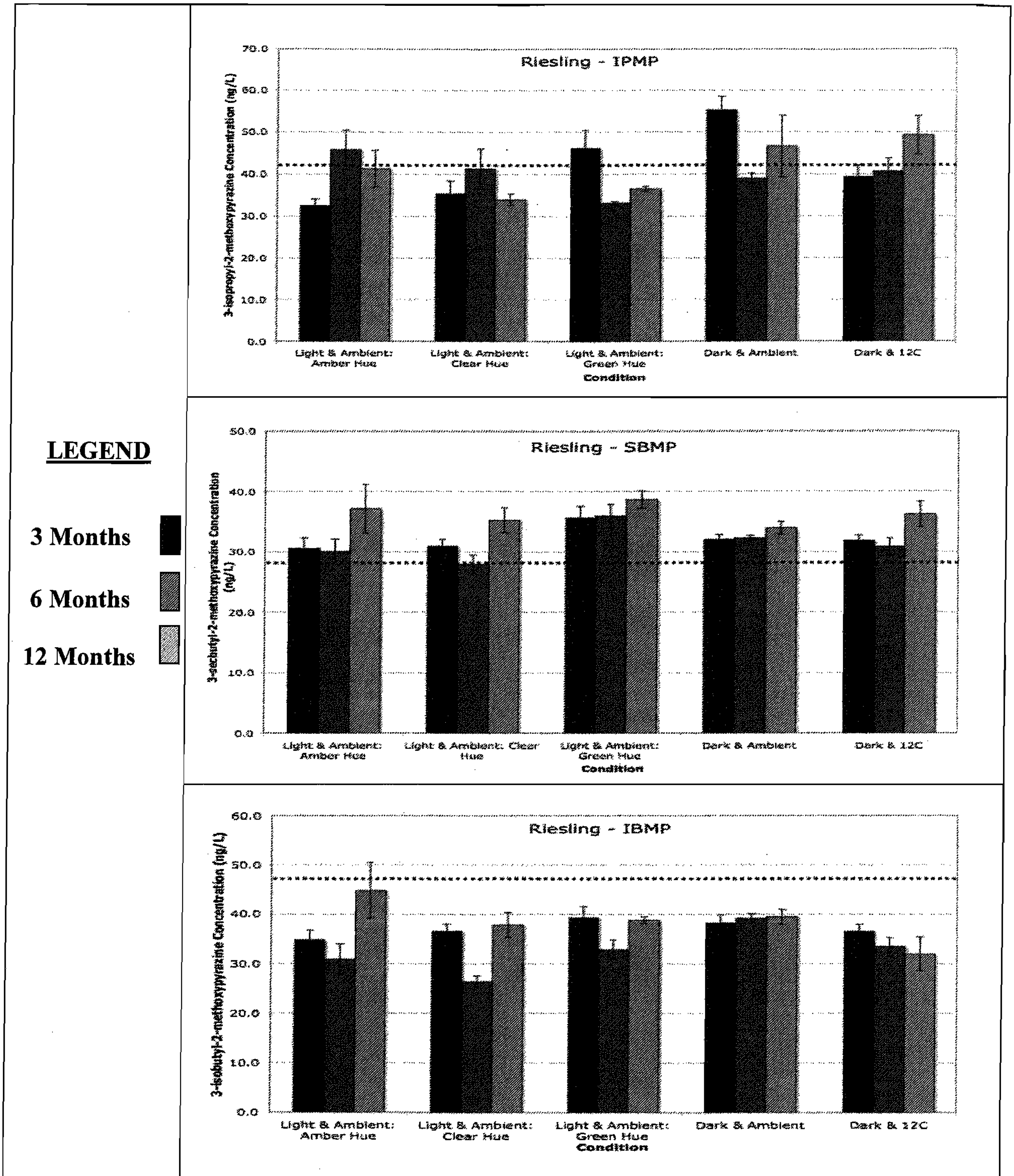


Figure 3-2: Concentration of 3-alkyl-2-methoxypyrazines (MP) in Riesling spiked with 30 ng/L of isopropyl-, *sec*butyl-, and isobutyl-MP. Data represent values of duplicate or triplicate measurements of duplicate bottles ±SEM. Means with letters are those which differ significantly. Dashed line indicates initial MP concentration at bottling.

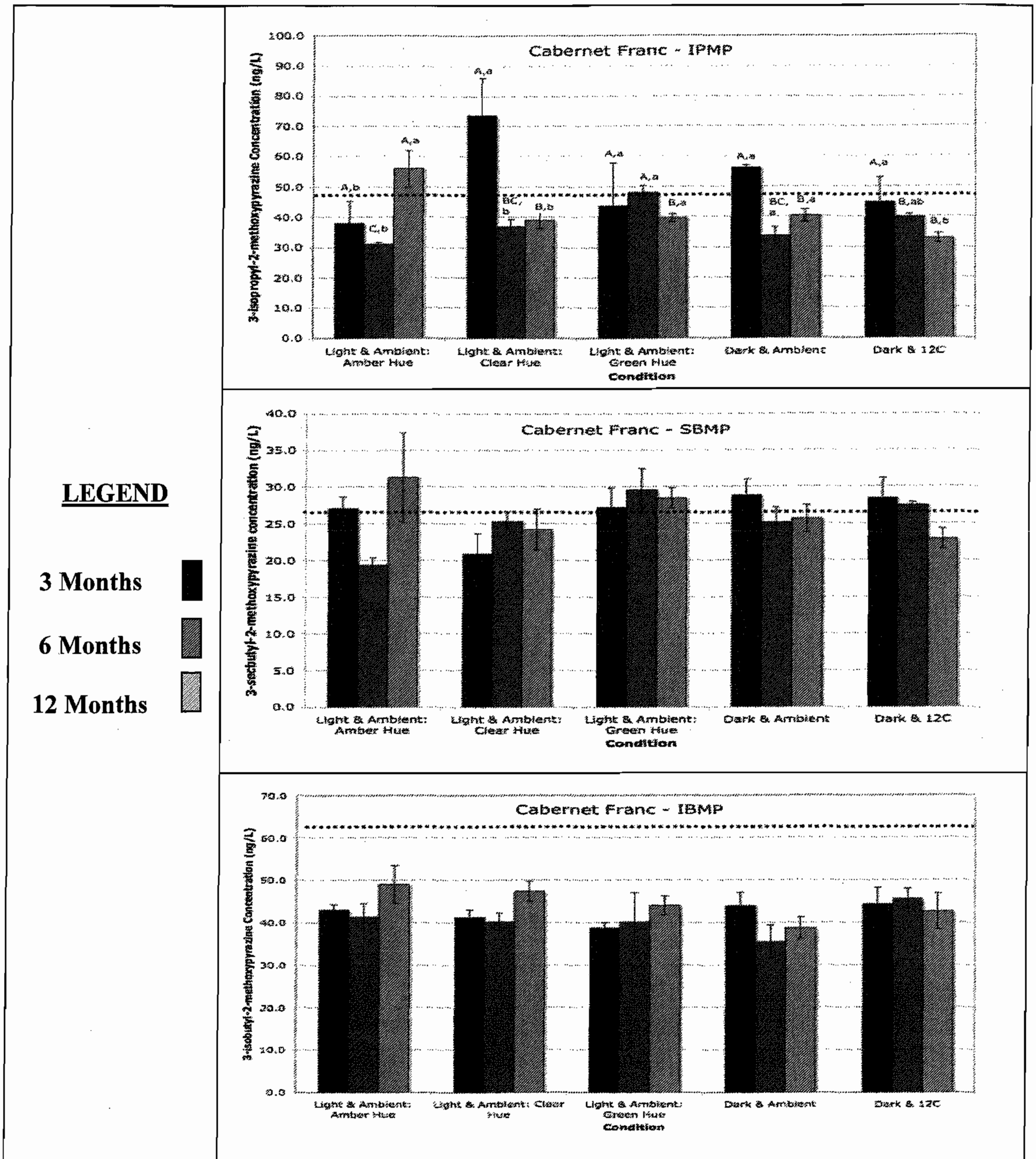


Figure 3-3: Concentration of 3-alkyl-2-methoxypyrazines (MP) in Cabernet Franc spiked with 30 ng/L of isopropyl-, *sec*butyl-, and isobutyl-MP. Data represent values of duplicate or triplicate measurements of duplicate bottles ±SEM. Means with letters are those which differ significantly, and of those, means sharing the same letter do not differ in groups across time [lowercase] or at specific time points [uppercase] (Fishers Protected $LSD_{0.05}$). Dashed line indicates initial MP concentration at bottling.

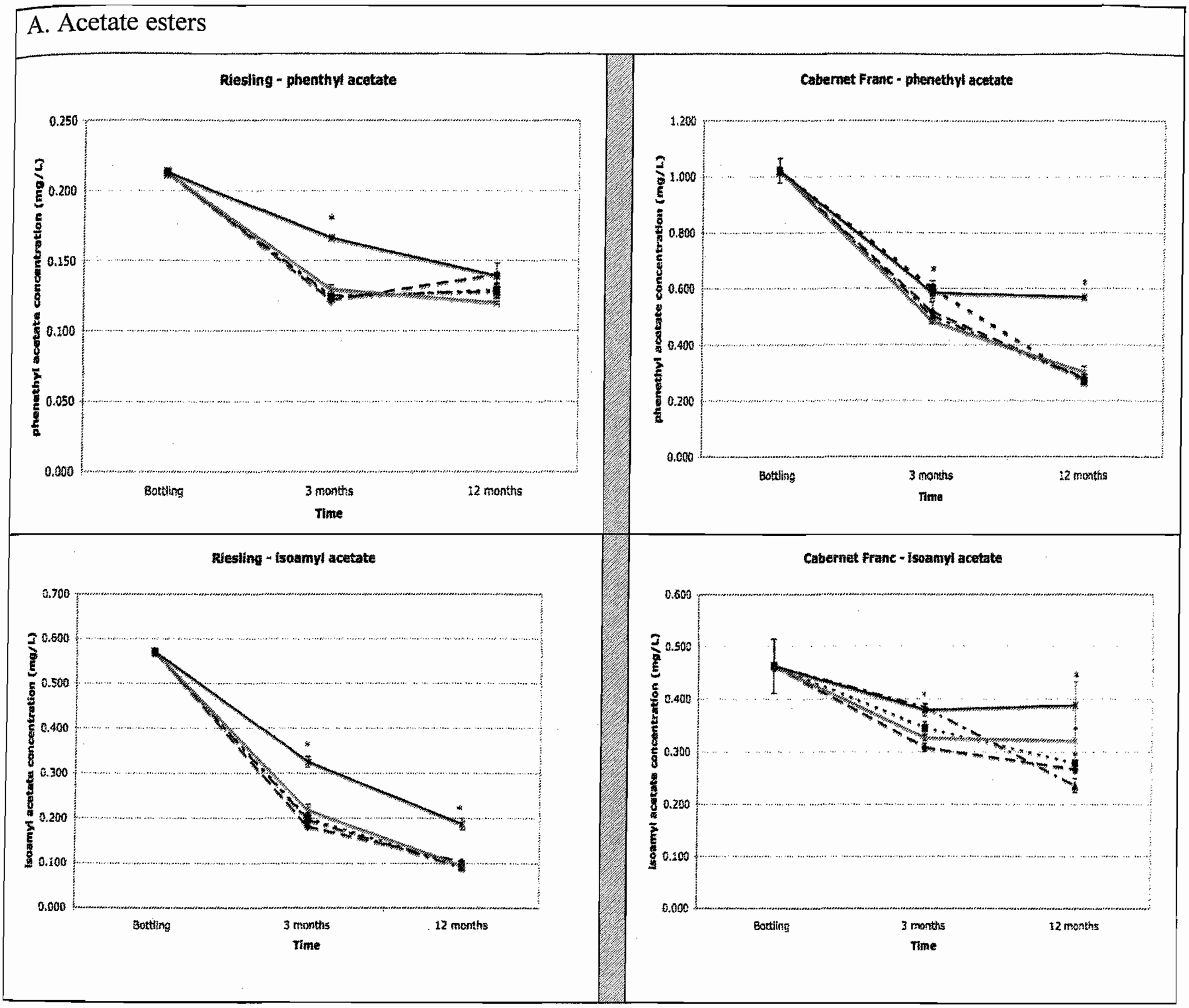


Figure 3-4: Indicator volatile acetate esters in Riesling and Cabernet Franc. Means that are significantly different (Fisher's Protected $LSD_{0.05}$) indicated with asterisk. Error bars represent ±SEM

LEGEND:
Light & Ambient, Amber Hue:
Light & Ambient, Clear Hue:
Light & Ambient, Green Hue:
Dark & Ambient:
Dark & Cellar:

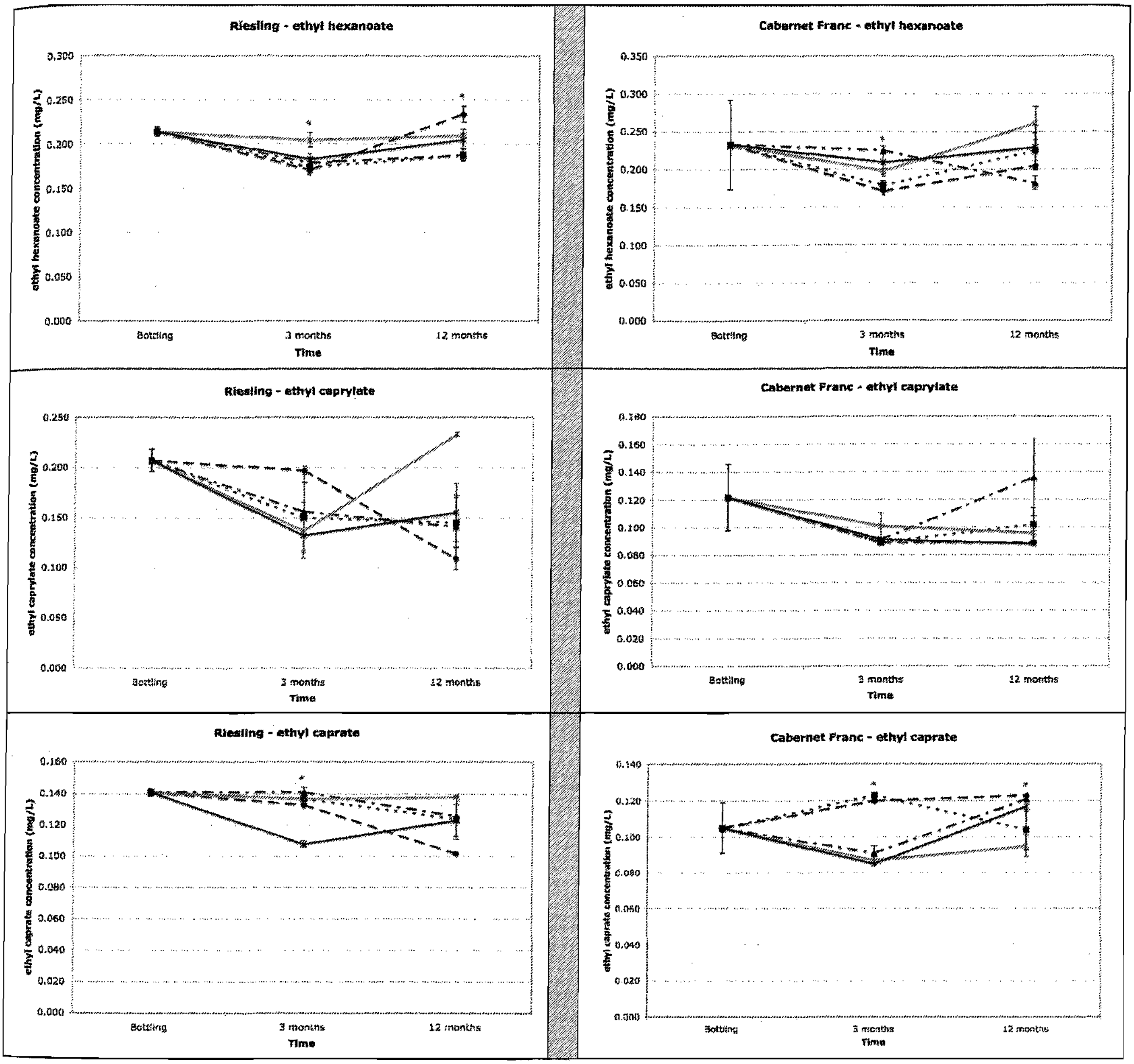


Figure 3-5: Indicator volatile ethyl esters in Riesling and Cabernet Franc. Means that are significantly different (Fisher's Protected $LSD_{0.05}$) indicated with asterisk. Error bars represent ±SEM

LEGEND:

Light & Ambient, Amber Hue:
Light & Ambient, Clear Hue:
Light & Ambient, Green Hue:
Dark & Ambient:
Dark & Cellar:

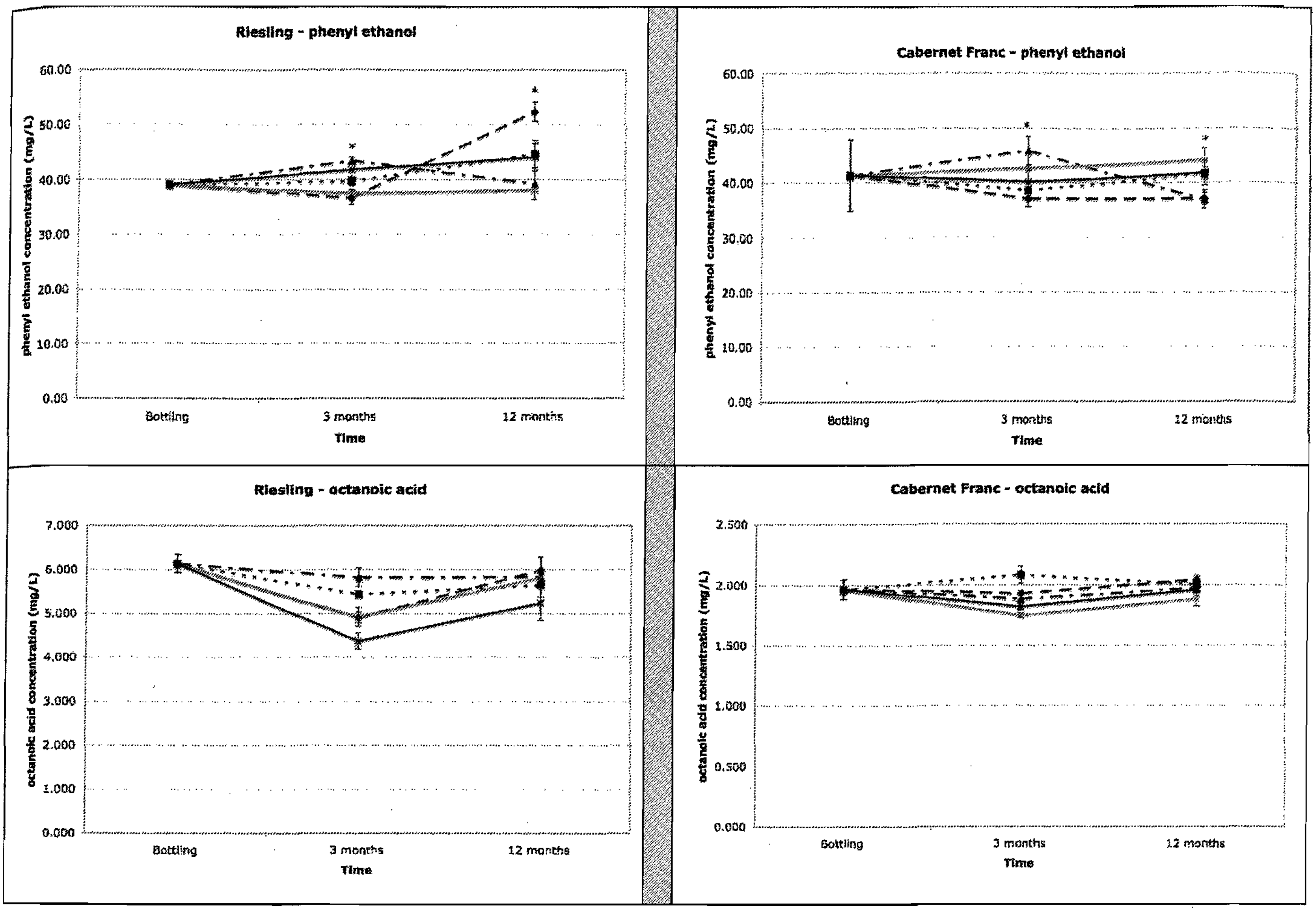


Figure 3-6: Indicator volatile alcohol and acid in Riesling and Cabernet Franc. Means that are significantly different (Fisher's Protected $LSD_{0.05}$) indicated with asterisk. Error bars represent ±SEM

LEGEND:
Light & Ambient, Amber Hue: - - - -
Light & Ambient, Clear Hue: ....
Light & Ambient, Green Hue: - · - · -
Dark & Ambient:
Dark & Cellar:

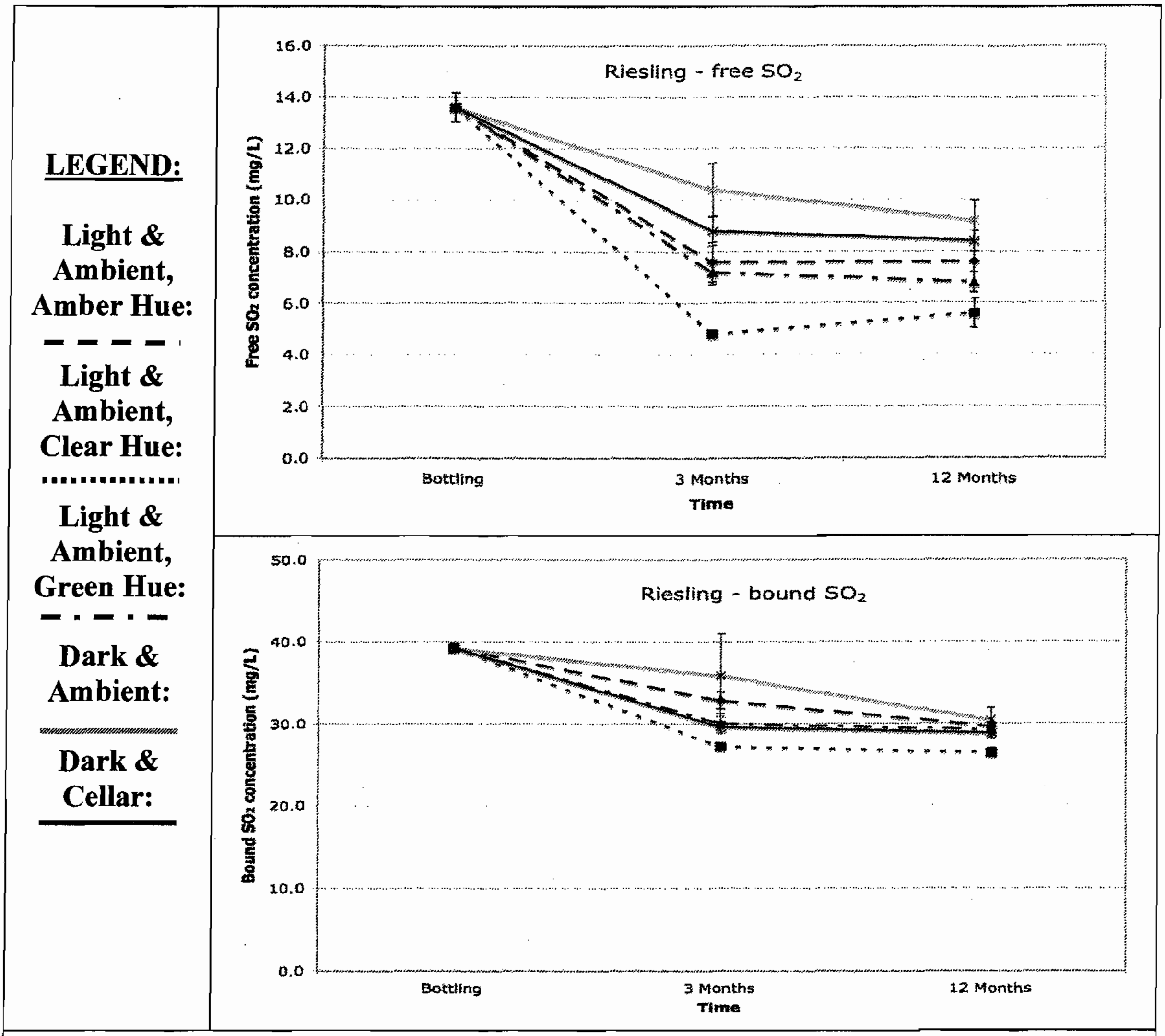


Figure 3-7: Free and Bound Sulfur Dioxide in Riesling wine. Data represent mean values of duplicate measurements of duplicate bottles ±SEM. An asterisk is above time points with significantly different means (Fisher's Protected $LSD_{0.05}$).

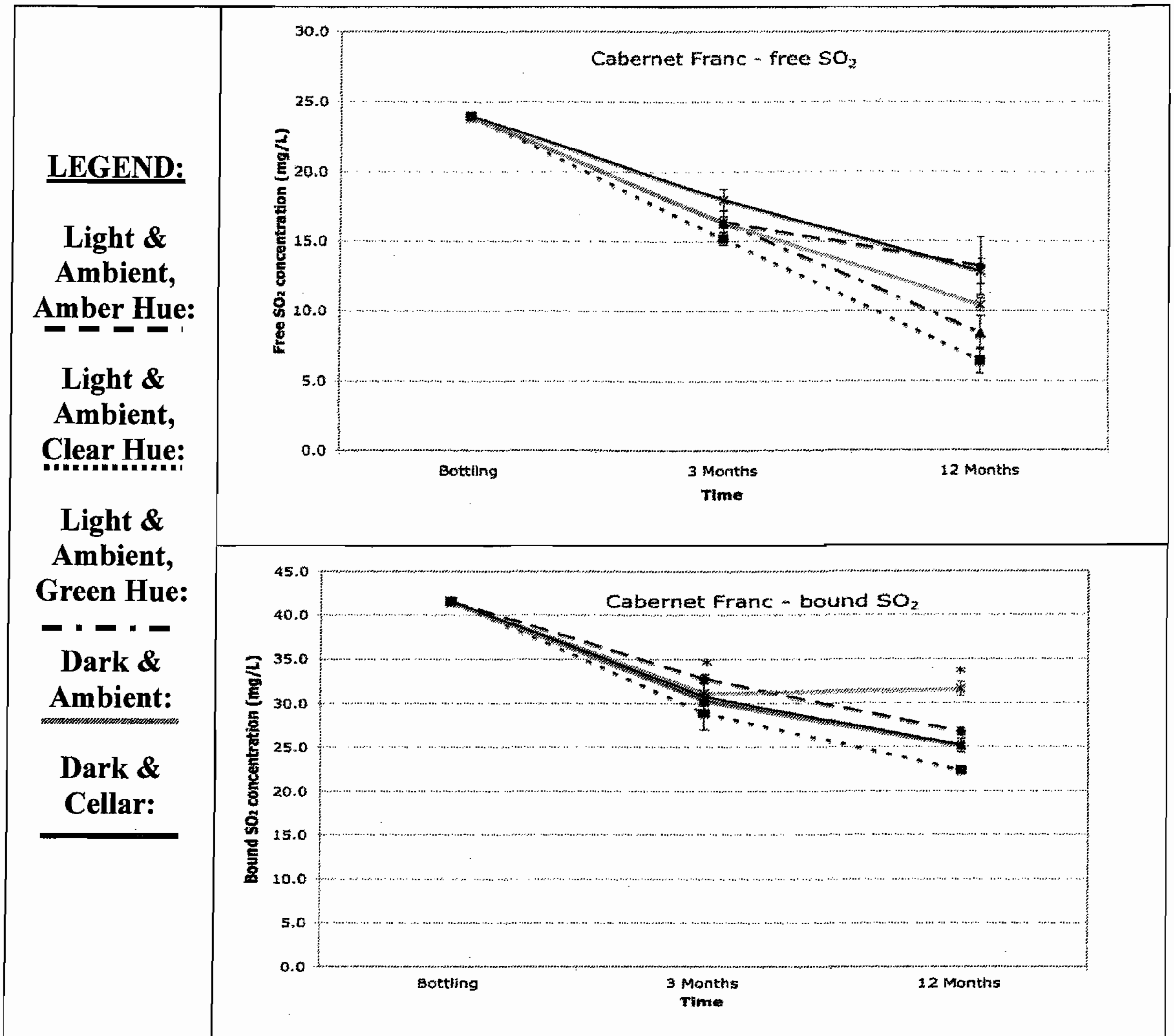
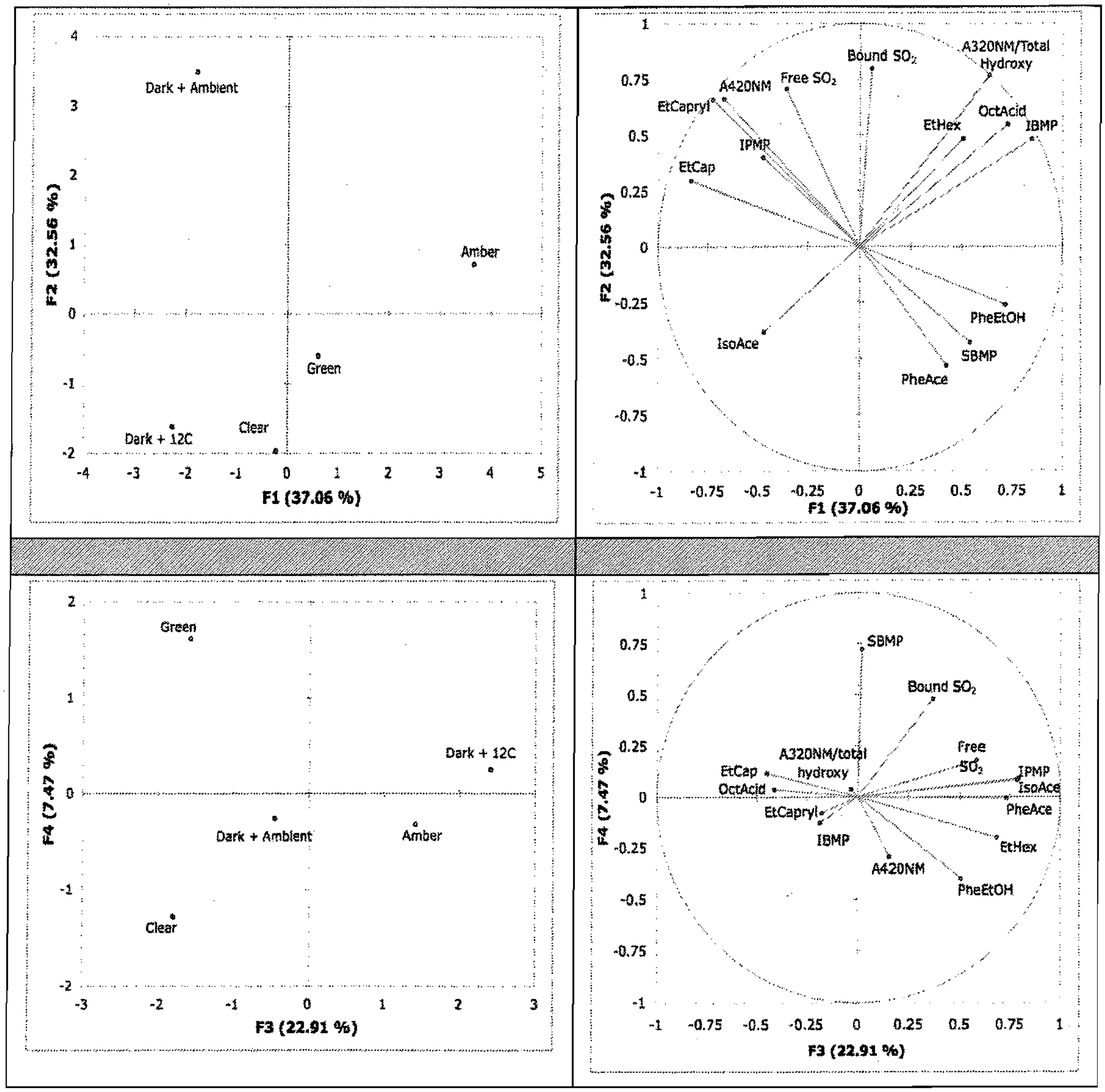
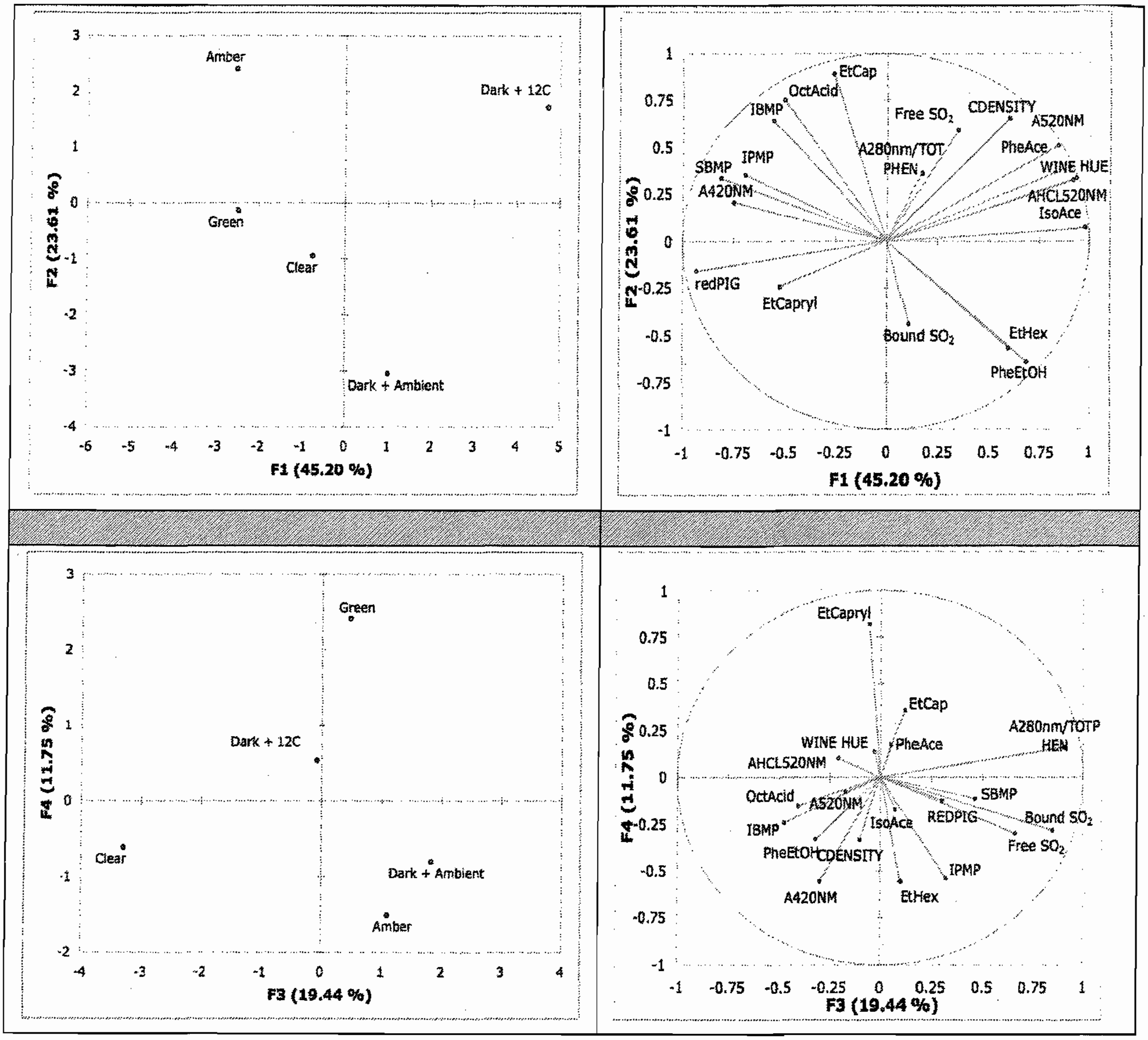


Figure 3-8: Free and Bound Sulfur Dioxide in Cabernet Franc wine. Data represent mean values of duplicate measurements of duplicate bottles ±SEM. An asterisk is above time points with significantly different means (Fisher's Protected $LSD_{0.05}$).



Abbreviation: IPMP: 3-isopropyl-2-methoxypyrazine; SBMP: 3-secbutyl-2-methoxypyrazine; IBMP: 3-isobutyl-2-methoxypyrazine; PheAce: phenethyl acetate; EtCap: ethyl caprate; EtCapryl: ethyl caprylate; EtHex: ethyl hexanoate; IsoAce: isoamyl acetate; PheEtOH: phenyl ethanol; OctAcid: octanoic acid

Figure 3-9: Principal Component 1 vs. 2 and Principal Component 3 vs. 4 for Riesling wine after 12 months.



Abbreviations: IPMP: 3-isopropyl-2-methoxypyrazine; SBMP: 3-secbutyl-2-methoxypyrazine; IBMP: 3-isobutyl-2-methoxypyrazine; PheAce: phenethyl acetate; EtCap: ethyl caprate; EtCapryl: ethyl caprylate; EtHex: ethyl hexanoate; IsoAce: isoamyl acetate; PheEtOH: phenyl ethanol; OctAcid: octanoic acid; CDensity: wine colour density; RedPig: Degree of Red Pigmentation

Figure 3-10: Principal Component 1 vs. 2 and Principal Component 3 vs. 4 for Cabernet Franc wine after 12 months.

Chapter 4

General Discussion and Conclusions

Volatile constituents in wine, those that elicit aromas, influence overall quality. 3-alkyl-2-methoxypyrazines (MPs), a class of compounds that educe "vegetative" or "herbaceous" percepts, adversely affect wine quality when present at elevated concentrations (1). MPs, specifically 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP) and 3-*sec*butyl-2-methoxypyrazine (SBMP) must be managed in order to achieve optimal wine sensory characteristics. The remediation of MPs in wine is made more challenging by their trace concentration (low ng/L range, regardless of source (1-3)) and extremely low sensory detection thresholds (as low as 320 pg/L (4)). Although, historically a significant barrier to MP research, the detection levels of modern analytical techniques now approach human sensitivity (5, 6) and have allowed for meaningful research on MPs in wine.

MPs are derived both from grapes and from exogenous sources. In grapes, MP levels decrease during ripening (1, 7, 8) and are relatively lower in warmer climates, as a consequence of increased light (8, 9) or temperature exposure (9). Hence, wine with elevated MP concentration or intense "herbaceous" quality is associated with sub-optimal, lower-quality fruit, and is of concern for grape growers who operate in climates where complete fruit maturation is compromised. Additionally, MPs, most abundantly IPMP, are derived from the haemolymph of the non-native coccinellid species, *Harmonia axyridis*, observed in Southern Ontario vineyards since 2001 (10). These ladybeetles, which aggregate in vineyards and become harvested with the grapes, contribute atypical "herbaceous" aromas and flavours to wine, termed "ladybug taint" (2). Ladybug taint prevention and remediation are focal topics in the local wine industry as LBT is responsible for great financial loss (11). The appearance and persistence of *Harmonia axyridis* in numerous wine regions around the world (12) suggest that elevated MPs or LBT-wine are or could be of global concern, as well.

Therefore for both grape-growers producing fruit in suboptimal climates or seasons and wine producers faced with LBT, mediation of MPs could produce extensive benefits. A review of relevant literature (*Chapter 1)* elucidated areas that hold promise for the control of MPs in wine: the direct and overall effect of wine packaging options (including wine closures) and the effect of light exposure and temperature conditions during storage. The former topic is known to affect other wine volatiles directly through a phenomenon termed "flavour scavenging" (the sorption of volatiles into or out of foodstuff (13)) and indirectly by mediating gas permeation into/out of wine; however, is untested for MPs. The second topic, related to conditions during storage, has been observed to cause MP degradation in grapes during ripening (8), in aqueous solution (14), but surprisingly, throughout peer-reviewed literature, has not been examined in wine.

For the current study it was first necessary to test whether contact with closure material could alter MP concentration (*Preliminary Trial)*. Closures immersed in MP-enriched wine provided a significant surface area to wine ratio. All closures decreased MP concentration to some extent with respect to controls over a relatively short period. Synthetic closure material resulted in the greatest MP decrease, especially for *sec*butyl-MP, and moulded synthetic closures had the highest adsorption. Plastic bottle tops used in this experiment were also responsible for a marginal MP decrease, which emphasizes the sorptive capacity of polymeric materials. These results encouraged further research into the effect of closure/packaging type on MPs in wine.

The closure/packaging longitudinal trial (*Chapter 2*) tested the hypothesis that closure and packaging options could alter MP concentration over 18 months in MP-enriched Riesling and Cabernet Franc wines packaged according to commercial practice. Additionally, impact odorants, and physico-chemical parameters of wine were measured for all treatments in order to gain a more complete understanding of how overall wine quality, a trait influenced by many factors, was being affected. Wines were stored under 6 closure options or packaged in TetraPak® cartons. All three MPs were affected by closure/packaging options. TetraPak® cartons were associated with the greatest MP decrease, followed by the moulded synthetic corks; while the highest final MP concentration was evident in the screwcap and/or natural cork-packaged wines (Riesling only). Interestingly, MPs (IPMP and SBMP) *increased* under some cork-based closures, implicating them for MP contribution or potentially implicating a yet unidentified wine constituent or phenomenon. In general, the impact odorants and other chemical constituents acted as expected from previous literature. TetraPak® cartons have not previously been tested for effects on wine volatiles. Data indicate that TetraPak® cartons allowed for increased oxygen ingress and perhaps decreased wine quality.

The second research objective was to explore the potential effect of elevated light exposure and temperature during storage on MPs (*Chapter 3*). Other analytes monitored in *Chapter 2* were again tested. Three chambers with distinct light and temperature conditions housed MP-enriched Cabernet Franc and Riesling wine over 12 months. After and during storage, MPs were not consistently affected by light or temperature exposure. This observation may have been obscured by MP migration from natural cork closures, as was detected in *Chapter 2*. Other analytes were affected by conditions, and reinforced the anecdotal information and practice of cooler temperature, light-excluded wine storage. Although the data suggest that light and temperature exposure do not directly affect MP concentration, it is possible that the noted influence of light/temperature on other constituents of wine, may indirectly affect the overall and MP-elicited percepts in wine.

These studies shared a common primary objective: examining potential treatments/conditions for the mediation of MPs in wine. Present empirical evidence suggests that closure and/or packaging type affects MP concentration. A possible next stage for this research is the generation of a curative treatment for MP-rich or LBT-wines. This research corroborated much of the accepted *best practice* for wine packaging and storage. The data also reinforce the importance of fully examining novel closure/packaging alternatives, such as TetraPak® cartons, which are commonly used throughout the global wine industry, yet barely reported on in peer-reviewed literature. Additionally, this research adds to the evidence for flavour scavenging of wine volatiles by natural and synthetic polymeric packaging. Only recently recognized and examined in wine (15), flavour scavenging deserves increased focus, including comprehensive research trials, like that conducted at the Australian Wine Research Institute. Finally, it should be noted that sensory difference thresholds for MPs in wine are presently unknown, and without them or direct sensory analysis on wines, potential perceivable changes corresponding to chemical changes are purely speculative.

The experimental results and discussions presented in preceding Chapters along with future research endeavors will provide valuable information and tools for oenologists, winemakers, flavour chemists and viticulturalists to achieve optimal wine quality. Secondarily, the present information contributes to the current understanding of 3-alkyl-2-methoxypyrazines; an interesting and elusive class of trace compounds.

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Appendix A – Supplemental data for Chapters 2,3

Table A-1: Riesling spectrophotometric parameters after 12 months closure/packaging trial (Chapter 2). Mean values are above ±SEM values, and followed by letters indicating statistical differences; letters with means that differ significantly are different (Fisher's Protected LSD (0.05)).

Parameter	Natural Cork	Agglomerate Cork	Synthetic Cork – Extruded	Synthetic Cork – Moulded	ScrewCap	Tetrapak
A280 nm	4.400bc ±0.122	4.600a ±0.020	4.350c ±0.056	4.658a ±0.021	4.630a ±0.020	4.440b ±0.025
A320 nm	2.910ab ±0.088	2.915ab ±0.023	2.848c ±0.036	2.955a ±0.005	2.943a ±0.055	2.878bc ±0.035
A420 nm	0.045b ±0.007	0.038b ±0.003	0.048b ±0.003	0.043b ±0.002	0.040b ±0.003	0.068a ±0.002
A520 nm	0.007b ±0.000	0.004c ±0.000	-0.001e ±0.001	0.008ab ±0.001	0.003d ±0.001	0.008a ±0.001
Total phenolics (A280 nm – 4)	0.40bc ±0.12	0.60a ±0.02	0.35c ±0.06	0.66a ±0.02	0.63a ±0.02	0.44b ±0.03
Total hydroxycinnamates (A320 nm – 1.4)	1.51ab ±0.09	1.52ab ±0.02	1.45c ±0.04	1.56a ±0.01	1.54a ±0.06	1.48bc ±0.04

Table A-2: Cabernet Franc spectrophotometric parameters after 12 months closure/packaging trial (Chapter 2). Mean values are above ±SEM values, and followed by letters indicating statistical differences; letters with means that differ significantly are different (Fisher's Protected LSD (0.05)).

Parameter	Natural Cork	Agglomerate Cork	Synthetic Cork – Extruded	Synthetic Cork – Moulded	ScrewCap	Tetrapak
A280 nm	42.243a ±0.195	45.829a ±1.030	45.526a ±3.996	41.057a ±2.670	45.147a ±1.809	44.314a ±0.655
A420 nm	1.355d ±0.049	1.778b ±0.094	1.573c ±0.031	1.623c ±0.011	1.528c ±0.089	2.218a ±0.074
A520 nm	1.795e ±0.032	2.395b ±0.165	1.990d ±0.032	2.083c ±0.017	2.103c ±0.155	2.858a ±0.089
Total phenolics (A280 nm – 4.000)	38.24a ±0.20	41.83a ±1.03	41.53a ±4.00	37.06a ±2.67	41.15a ±1.81	40.31a ±0.66
Wine hue (A520 nm/A420 nm)	0.75abc ±0.01	0.75bc ±0.01	0.79a ±0.00	0.78ab ±0.00	0.73c ±0.03	0.78ab ±0.00
Wine colour density (A520 nm + A420 nm)	3.15e ±0.08	4.17b ±0.26	3.56d ±0.06	3.71c ±0.03	3.63cd ±0.23	5.08a ±0.16
Degree of red pigmentation (A520 nm/AHCl520 nm)*100	22.67c ±1.07	29.14b ±1.53	24.39c ±1.65	26.33bc ±2.34	24.685c ±0.88	50.71a ±3.53

Table A-3: Riesling spectrophotometric parameters after 12 months light/temperature trial (Chapter 3). Mean values are above ±SEM values, and followed by letters indicating statistical differences; letters with means that differ significantly are different (Fisher's Protected LSD (0.05)).

RIESLING	AMBER	CLEAR	GREEN	DARK & AMBIENT	DARK & 12C
A280NM	*	4.910a ±0.040	5.050a ±0.030	4.958a ±0.056	4.103b ±0.028
A320NM	3.070a ±0.020	2.760a ±0.010	2.885a ±0.015	2.995a ±0.038	2.688a ±0.149
A420NM	0.036a ±0.003	0.037a ±0.002	0.035a ±0.002	0.043a ±0.002	0.039a ±0.004
A520NM	*	0.000a ±0.000	0.004a ±0.000	0.007a ±0.000	0.002a ±0.000
TOTAL PHENOLICS (A280 – 4)	*	0.91a ±0.04	1.05a ±0.03	0.96a ±0.06	0.10b ±0.03
TOTAL HYDROXYCINNAMATES (A320 – 1.4)	1.67a ±0.02	1.36a ±0.01	1.49a ±0.02	1.60a ±0.04	1.29a ±0.15

* Note: This sample was not available at the time of analysis

Table A-4: Cabernet Franc spectrophotometric parameters after 12 months light/temperature trial (Chapter 3). Mean values are above ±SEM values, and followed by letters indicating statistical differences; letters with means that differ significantly are different (Fisher's Protected LSD (0.05)).

Cabernet Franc	AMBER	CLEAR	GREEN	DARK & AMBIENT	DARK & 12C
AHCL280NM	41.789a ±0.932	38.102a ±2.252	41.334a ±0.581	41.334a ±0.612	41.764a ±0.593
A420NM	1.495a ±0.005	1.478a ±0.013	1.430ab ±0.01	1.423b ±0.028	1.395b ±0.003
AHCL520NM	4.798b ±0.152	5.656b ±0.846	4.747b ±0.101	5.151b ±0.289	8.358a ±0.145
A520NM	1.680b ±0.01	1.685b ±0.023	1.605b ±0.015	1.640b ±0.035	1.873a ±0.007
TOTAL PHENOLICS (A280 – 4)	37.79a ±0.93	34.10a ±2.25	37.33a ±0.58	37.33a ±0.61	37.76a ±0.59
WINE COLOUR DENSITY (A420 + A520)	3.18ab ±0.02	3.16ab ±0.04	3.04b ±0.03	3.06b ±0.06	3.27a ±0.01
WINE HUE (A420/A520)	0.89a ±0.00	0.88b ±0.00	0.89a ±0.00	0.87c ±0.00	0.75d ±0.00
DEG RED PIG ((A520/AHCl520)*100)	35.06a ±1.32	31.60a ±4.07	33.83a ±1.04	32.13a ±1.82	22.43b ±0.44

Table A-5: pH level and titratable acidity of samples at bottling, and after 12 months storage (Chapter 3, 4)

WINES:	Riesling		Cabernet Franc	
Treatment	Average pH	Average titratable acidity (g/L)	Average pH	Average titratable acidity (g/L)
Bottling	2.832 ±0.039	7.88 ±0.38	3.720 ±0.000	4.1 ±0.0
Natural Cork	2.854 ±0.073	7.35 ±0.74	3.656 ±0.000	3.8 ±0.0
Agglomerate Cork	2.761 ±0.012	7.57 ±0.60	3.691 ±0.000	3.9 ±0.0
Synthetic - Extruded	2.760 ±0.086	7.73 ±0.06	3.612 ±0.034	4.0 ±0.1
Synthetic - Moulded	2.806 ±0.083	7.67 ±0.20	3.701 ±0.025	4.1 ±0.1
Screw Cap	2.802 ±0.018	7.69 ±0.32	3.699 ±0.000	4.2 ±0.0
Tetrapak	2.783 ±0.018	7.72 ±0.17	3.708 ±0.005	4.2 ±0.0
Amber	2.879 ±0.072	6.98 ±0.18	3.682 ±0.000	4.1 ±0.0
Clear	2.816 ±0.000	6.94 ±0.00	3.700 ±0.010	4.2 ±0.0
Green	2.953 ±0.000	7.43 ±0.00	3.704 ±0.000	4.2 ±0.0
dark + ambient	2.864 ±0.008	7.33 ±0.18	3.700 ±0.012	4.2 ±0.1
dark + cellar	2.895 ±0.057	7.57 ±0.73	3.687 ±0.012	4.3 ±0.0

Table A-6: ANCOVA tables for Chapters 2 & 3

Chapter 2:

Riesling IPMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	74.891	74.891	0.911	0.342
Closure	5	1292.828	258.566	3.146	0.012
Time (weeks)*Closure	5	559.114	111.823	1.360	0.247

Riesling SBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	11.962	11.962	0.312	0.578
Closure	5	3581.475	716.295	18.697	< 0.0001
Time (weeks)*Closure	5	1285.848	257.170	6.713	< 0.0001

Riesling IBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	354.884	354.884	5.775	0.018
Closure	5	1060.324	212.065	3.451	0.007
Time (Weeks)*Closure	5	911.343	182.269	2.966	0.016

Cabernet Franc IPMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	154.196	154.196	3.485	0.065
Closure	5	661.665	132.333	2.991	0.016
Time (weeks)*Closure	5	86.009	17.202	0.389	0.855

Cabernet Franc SBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	115.430	115.430	3.649	0.061
Closure	5	142.342	28.468	0.900	0.487
Time (weeks)*Closure	5	202.916	40.583	1.283	0.283

Cabernet Franc - IBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	13951.49 7	13951.49 7	436.09 1	< 0.0001
Closure	5	2900.601	580.120	18.133	< 0.0001
Time (weeks)*Closure	5	428.040	85.608	2.676	0.027

Riesling - Phenethyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	0.000	0.000	0.523	0.474
Closure	5	0.001	0.000	3.442	0.012
Time (Weeks)*Closure	5	0.001	0.000	2.543	0.046

Riesling - Ethyl Caprate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	0.001	0.001	15.767	0.000
Closure	5	0.003	0.001	7.647	< 0.0001
Time (Weeks)*Closure	5	0.002	0.000	4.549	0.003

Riesling - Ethyl Caprylate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	0.028	0.028	11.273	0.002
Closure	5	0.067	0.013	5.351	0.001
Time (Weeks)*Closure	5	0.080	0.016	6.433	0.000

Riesling - Ethyl Hexanoate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	0.003	0.003	3.944	0.055
Closure	5	0.003	0.001	0.866	0.513
Time (Weeks)*Closure	5	0.005	0.001	1.179	0.339

Riesling - Isoamyl Acetate:

Source	DF	Sum of	Mean	F	Pr > F

		squares	squares		
Time (Weeks)	1	0.089	0.089	50.302	< 0.0001
Closure	5	0.033	0.007	3.665	0.009
Time (Weeks)*Closure	5	0.018	0.004	2.068	0.093

Riesling - Phenyl Ethanol:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	8.782	8.782	0.644	0.428
Closure	5	14.739	2.948	0.216	0.953
Time (Weeks)*Closure	5	87.329	17.466	1.280	0.295

Riesling - Octanoic Acid:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (Weeks)	1	2.799	2.799	11.577	0.002
Closure	5	8.658	1.732	7.162	0.000
Time (Weeks)*Closure	5	8.923	1.785	7.382	< 0.0001

Cabernet Franc - Phenethyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	2.287	2.287	430.306	< 0.0001
Closure	5	0.060	0.012	2.270	0.068
Times (weeks)*Closure	5	0.121	0.024	4.544	0.003

Cabernet Franc - Ethyl Caprate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	0.010	0.010	323.698	< 0.0001
Closure	5	0.000	0.000	1.234	0.313
Times (weeks)*Closure	5	0.002	0.000	10.809	< 0.0001

Cabernet Franc - Ethyl Caprylate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	0.045	0.045	25.419	< 0.0001
Closure	5	0.049	0.010	5.499	0.001
Times (weeks)*Closure	5	0.029	0.006	3.228	0.016

Cabernet Franc - Ethyl Hexanoate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	0.004	0.004	6.041	0.019
Closure	5	0.021	0.004	6.303	0.000
Times (weeks)*Closure	5	0.002	0.000	0.572	0.721

Cabernet Franc - Isoamyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	0.050	0.050	15.650	0.000
Closure	5	0.041	0.008	2.526	0.047
Times (weeks)*Closure	5	0.041	0.008	2.553	0.045

Cabernet Franc - Phenyl Ethanol:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	49.616	49.616	2.329	0.136
Closure	5	115.553	23.111	1.085	0.385
Times (weeks)*Closure	5	146.741	29.348	1.377	0.256

Cabernet Franc - Octanoic Acid:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Times (weeks)	1	0.373	0.373	15.650	0.000
Closure	5	0.411	0.082	3.455	0.012
Times (weeks)*Closure	5	0.260	0.052	2.186	0.077

Riesling - Free SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	32.000	32.000	13.483	0.001
Closure	5	58.250	11.650	4.909	0.001
Time (weeks)*Closure	5	86.968	17.394	7.328	< 0.0001

Riesling - Bound SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	15.680	15.680	1.331	0.256
CLOSURE	5	76.201	15.240	1.294	0.287

Time (weeks)*CLOSURE	5	595.472	119.094	10.110	< 0.0001

Cabernet Franc - Free SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	11.520	11.520	3.337	0.076
CLOSURE	5	560.144	112.029	32.447	< 0.0001
Time (weeks)*CLOSURE	5	73.662	14.732	4.267	0.004

Cabernet Franc - Bound SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	15.680	15.680	0.800	0.377
CLOSURE	5	703.319	140.664	7.178	< 0.0001
Time (weeks)*CLOSURE	5	140.276	28.055	1.432	0.235

Chapter 3:

Riesling - IPMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	101.768	101.768	1.470	0.231
Light/Temp	4	556.173	139.043	2.008	0.109
Time (weeks)*Light/Temp	4	523.932	130.983	1.892	0.128

Riesling - SBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	206.515	206.515	15.250	0.000
Light/Temp	4	200.083	50.021	3.694	0.010
Time (weeks)*Light/Temp	4	33.291	8.323	0.615	0.654

Riesling - IBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	67.667	67.667	2.203	0.144
Light/Temp	4	243.339	60.835	1.980	0.112
Time (weeks)*Light/Temp	4	276.173	69.043	2.248	0.077

Cabernet Franc - IPMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	919.568	919.568	7.056	0.011
Light/Temp	4	1831.559	457.890	3.513	0.014
Time (weeks)*Light/Temp	4	2054.514	513.629	3.941	0.008

Cabernet Franc - SBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	95.536	95.536	3.376	0.073
Light/Temp	4	183.643	45.911	1.623	0.185
Time (weeks)*Light/Temp	4	183.314	45.828	1.620	0.185

Cabernet Franc - IBMP:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	93.839	93.839	2.094	0.154
Light/Temp	4	152.938	38.235	0.853	0.499
Time (weeks)*Light/Temp	4	225.504	56.376	1.258	0.300

Riesling - Phenethyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.001	0.001	10.090	0.004
Condition	4	0.005	0.001	18.071	< 0.0001
Time (weeks)*Condition	4	0.002	0.001	8.712	0.000

Riesling - Ethyl Caprate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.002	0.002	9.569	0.004
Condition	4	0.003	0.001	3.442	0.021
Time (weeks)*Condition	4	0.002	0.001	3.063	0.033

Riesling - Ethyl Caprylate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.015	0.015	5.934	0.021
Condition	4	0.018	0.004	1.713	0.175

Time (weeks)*Condition	4	0.029	0.007	2.793	0.045

Riesling - Ethyl Hexanoate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.008	0.008	26.204	< 0.0001
Condition	4	0.003	0.001	2.477	0.067
Time (weeks)*Condition	4	0.004	0.001	3.431	0.021

Riesling - Isoamyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.013	0.013	40.247	< 0.0001
Condition	4	0.035	0.009	28.132	< 0.0001
Time (weeks)*Condition	4	0.004	0.001	3.043	0.033

Riesling - Phenyl Ethanol:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	494.199	494.199	40.303	< 0.0001
Condition	4	206.842	51.710	4.217	0.009
Time (weeks)*Condition	4	429.086	107.272	8.748	0.000

Riesling - Octanoic Acid:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	2.333	2.333	11.269	0.002
Condition	4	4.130	1.033	4.988	0.004
Time (weeks)*Condition	4	1.740	0.435	2.101	0.107

Cabernet Franc - Phenethyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.387	0.387	275.730	< 0.0001
Condition	4	0.201	0.050	35.747	< 0.0001
Time (weeks)*Condition	4	0.106	0.027	18.870	< 0.0001

Cabernet Franc - Ethyl Caprate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.001	0.001	16.396	0.000

Condition	4	0.004	0.001	15.165	< 0.0001
Time (weeks)*Condition	4	0.003	0.001	12.119	< 0.0001

Cabernet Franc - Ethyl Caprylate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.001	0.001	2.438	0.129
Condition	4	0.003	0.001	1.959	0.126
Time (weeks)*Condition	4	0.003	0.001	2.094	0.106

Cabernet Franc - Ethyl Hexanoate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.006	0.006	6.010	0.020
Condition	4	0.008	0.002	2.171	0.096
Time (weeks)*Condition	4	0.013	0.003	3.505	0.018

Cabernet Franc - Isoamyl Acetate:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.025	0.025	18.427	0.000
Condition	4	0.042	0.010	7.619	0.000
Time (weeks)*Condition	4	0.031	0.008	5.683	0.002

Cabernet Franc - Phenyl Ethanol:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	2.606	2.606	0.196	0.661
Condition	4	167.006	41.752	3.147	0.028
Time (weeks)*Condition	4	181.285	45.321	3.416	0.020

Cabernet Franc - Octanoic Acid:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	0.062	0.062	7.028	0.013
Condition	4	0.243	0.061	6.848	0.000
Time (weeks)*Condition	4	0.066	0.017	1.863	0.143

Riesling - Free SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F

Time (weeks)	1	0.000	0.000	0.000	1.000
CONDiTION	4	31.435	7.859	4.995	0.004
Time (weeks)*CONDiTION	4	3.021	0.755	0.480	0.750

Riesling - Bound SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	20.480	20.480	1.304	0.265
CONDiTION	4	105.341	26.335	1.677	0.188
Time (weeks)*CONDiTION	4	30.908	7.727	0.492	0.742

Cabernet Franc - Free SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	389.376	389.376	102.539	< 0.0001
CONDiTION	4	109.696	27.424	7.222	0.000
Time (weeks)*CONDiTION	4	40.064	10.016	2.638	0.053

Cabernet Franc - Bound SO2:

Source	DF	Sum of squares	Mean squares	F	Pr > F
Time (weeks)	1	210.681	210.681	46.273	< 0.0001
CONDiTION	4	150.516	37.629	8.265	0.000
Time (weeks)*CONDiTION	4	64.244	16.061	3.528	0.018

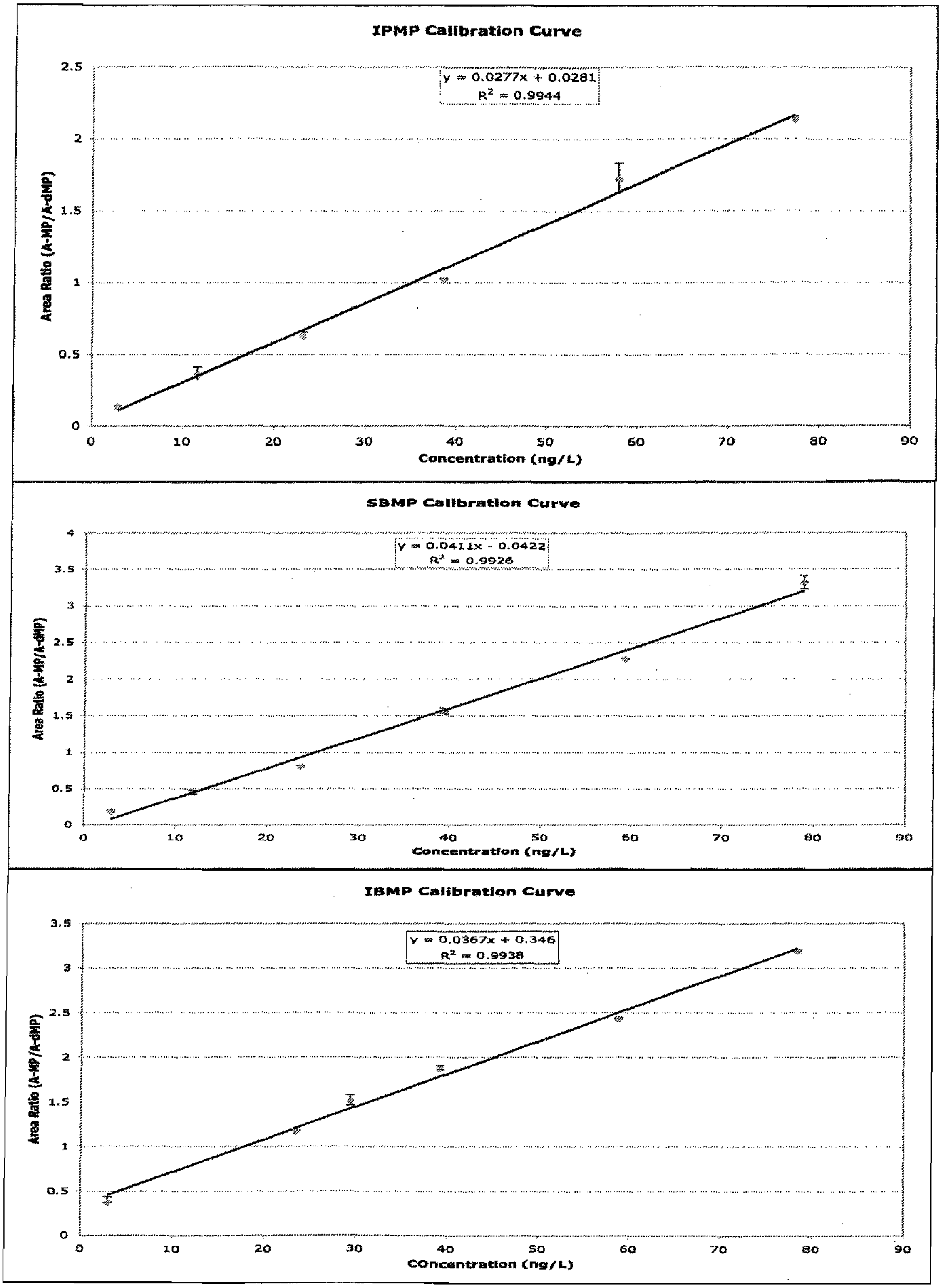


Figure A-1: Methoxypyrazine (IPMP, SBMP, IBMP) calibration curves for 12 months analysis (Chapter 2) and all analysis (Chapter 3). Data represent mean values of duplicate standards ±SD

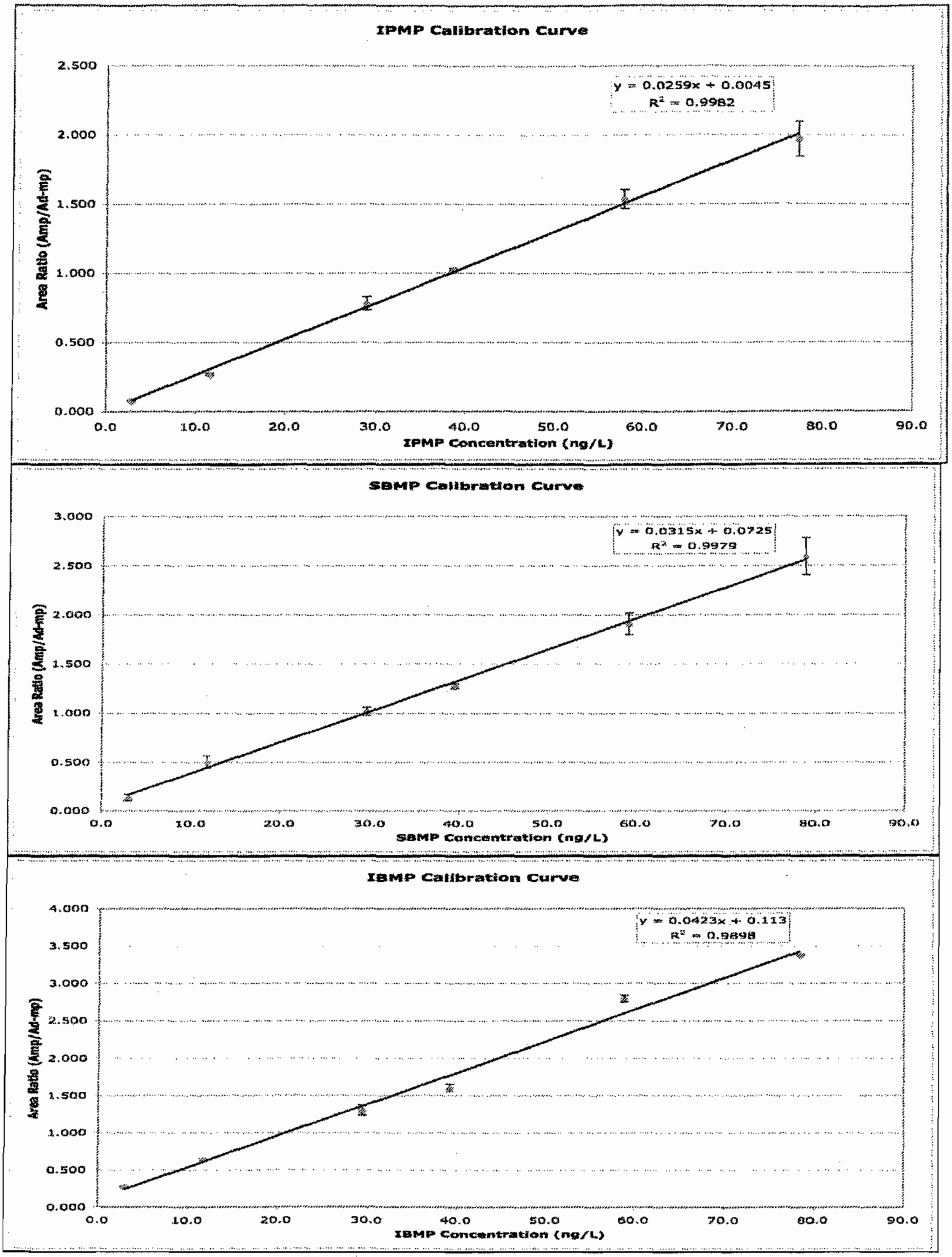
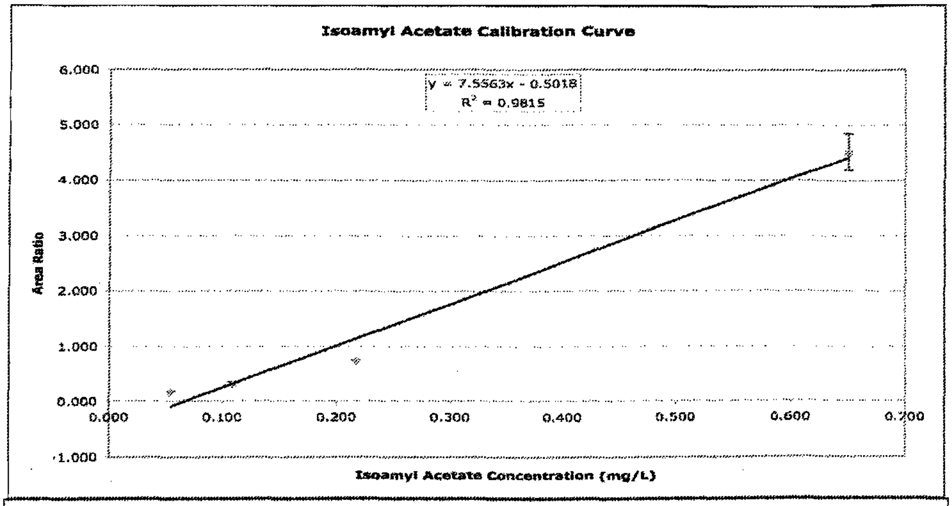
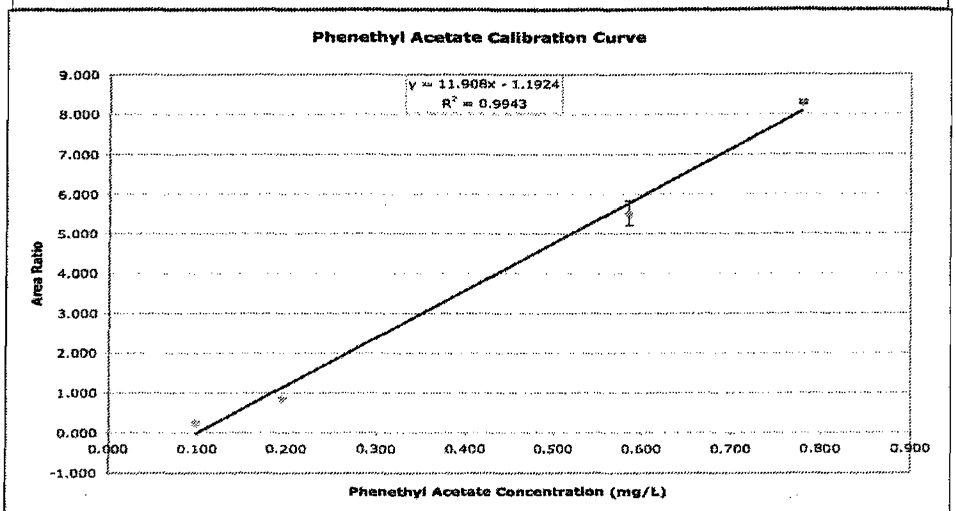
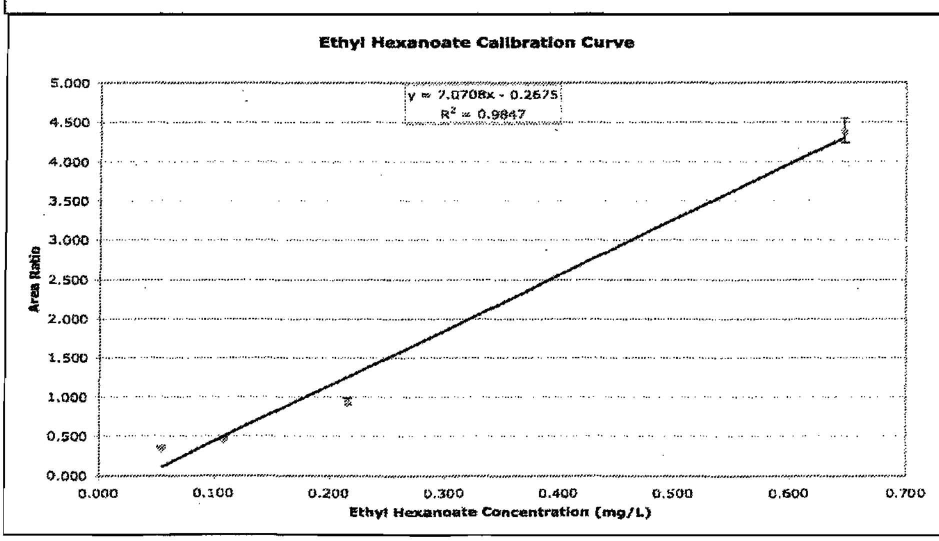
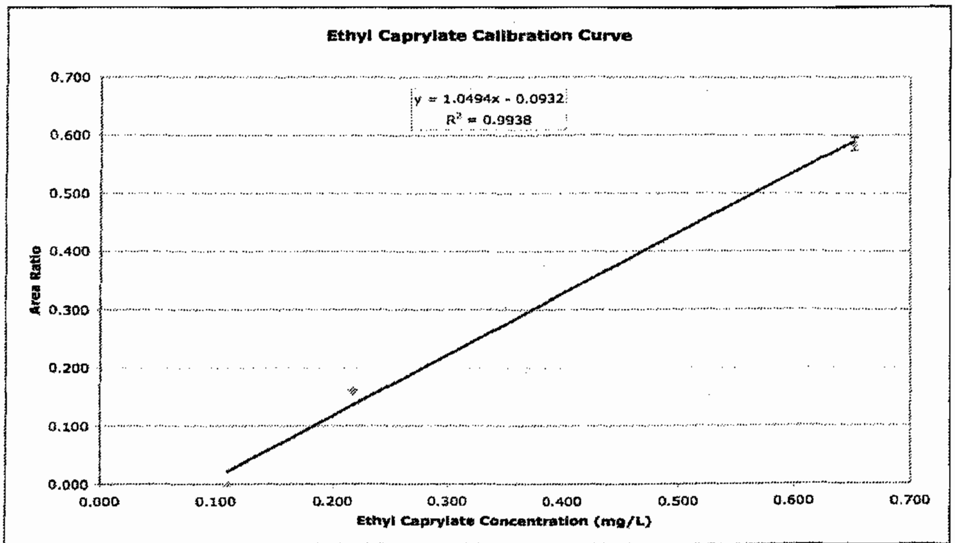


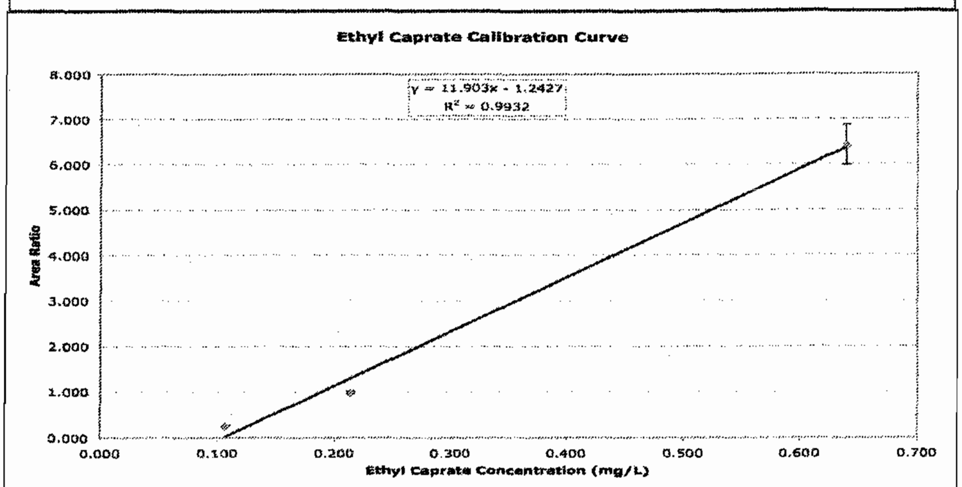
Figure A-2: Methoxypyrazine calibration curves for 18 months analysis (Chapter 2). Data represent mean values of duplicate standards ±SD

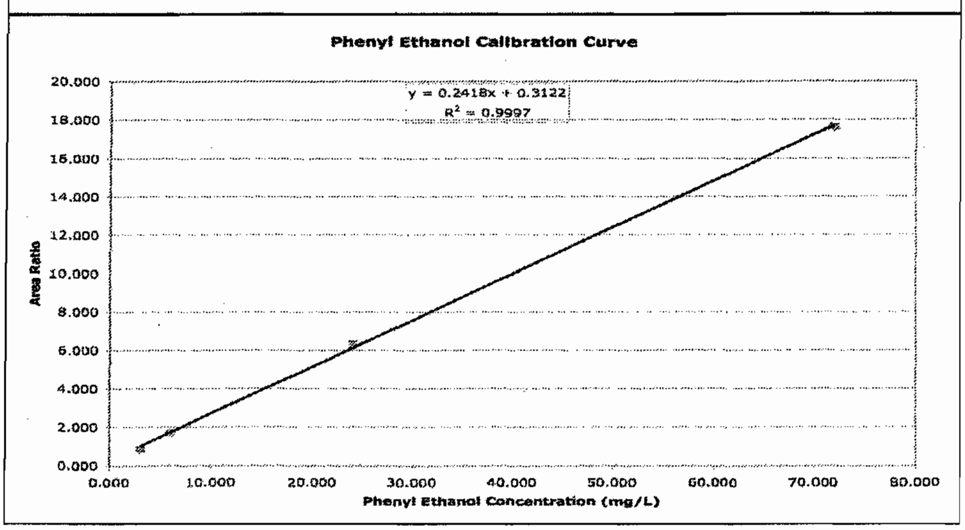

Isoamyl Acetate Calibration Curve
y = 7.5563x - 0.5018
R² = 0.9815
Area Ratio
Isoamyl Acetate Concentration (mg/L)


Phenethyl Acetate Calibration Curve
y = 11.908x - 1.1924
R² = 0.9943
Area Ratio
Phenethyl Acetate Concentration (mg/L)


Ethyl Hexanoate Calibration Curve
y = 7.0708x - 0.2675
R² = 0.9847
Area Ratio
Ethyl Hexanoate Concentration (mg/L)


Ethyl Caprylate Calibration Curve
y = 1.0494x - 0.0932
R² = 0.9938
Area Ratio
0.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
Ethyl Caprylate Concentration (mg/L)


Ethyl Caprate Calibration Curve
y = 11.903x - 1.2427
R² = 0.9932
Area Ratio
0.000
1.000
2.000
3.000
4.000
5.000
6.000
7.000
8.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
Ethyl Caprate Concentration (mg/L)


Phenyl Ethanol Calibration Curve
y = 0.2418x + 0.3122
R² = 0.9997
Area Ratio
0.000
2.000
4.000
6.000
8.000
10.000
12.000
14.000
16.000
18.000
20.000
30.000
40.000
50.000
60.000
70.000
80.000
Phenyl Ethanol Concentration (mg/L)

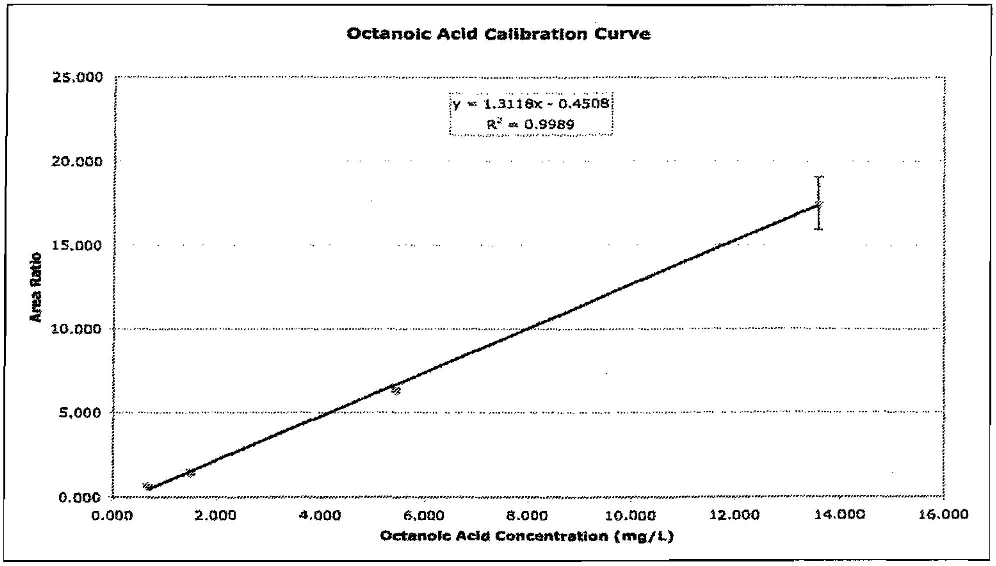


Figure A-3: Indicator volatile calibration curves for calibration (1 example of curves, repeated throughout analysis. Data represent mean values of duplicate standards ±SD

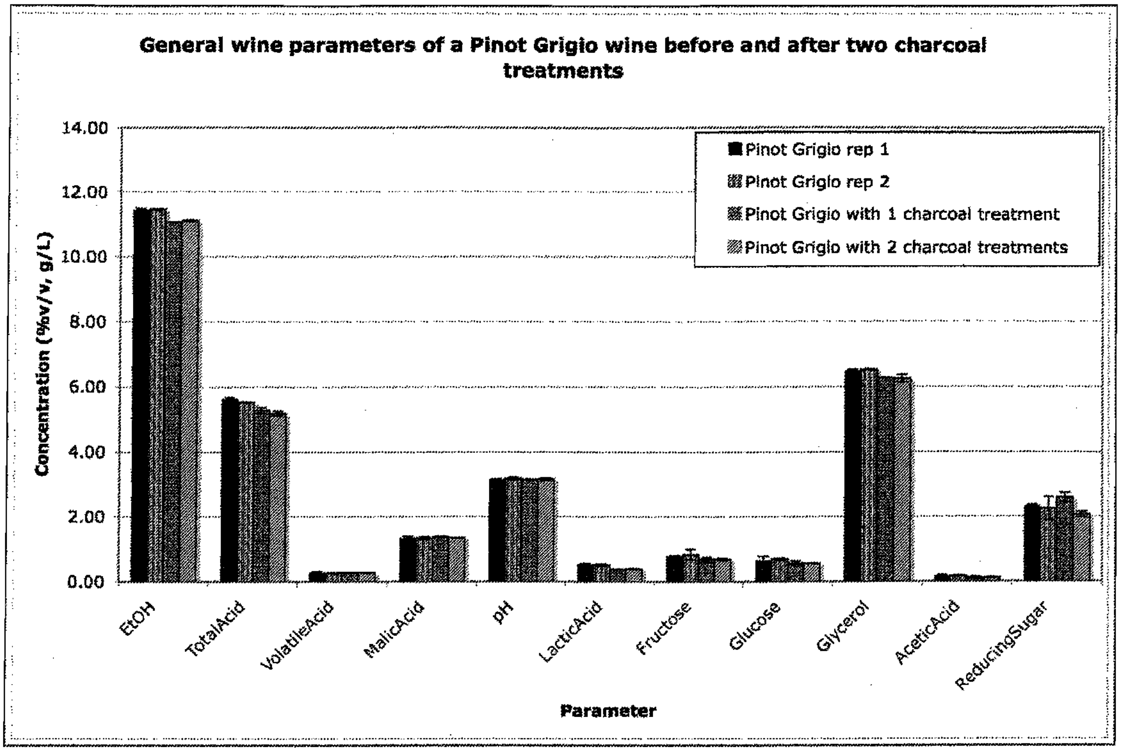


Figure A-4: Wine SCAN data before and after charcoal treatments during wine deodorizing (Chapter 2 and 3). Data represent mean values of duplicate standards ±SD

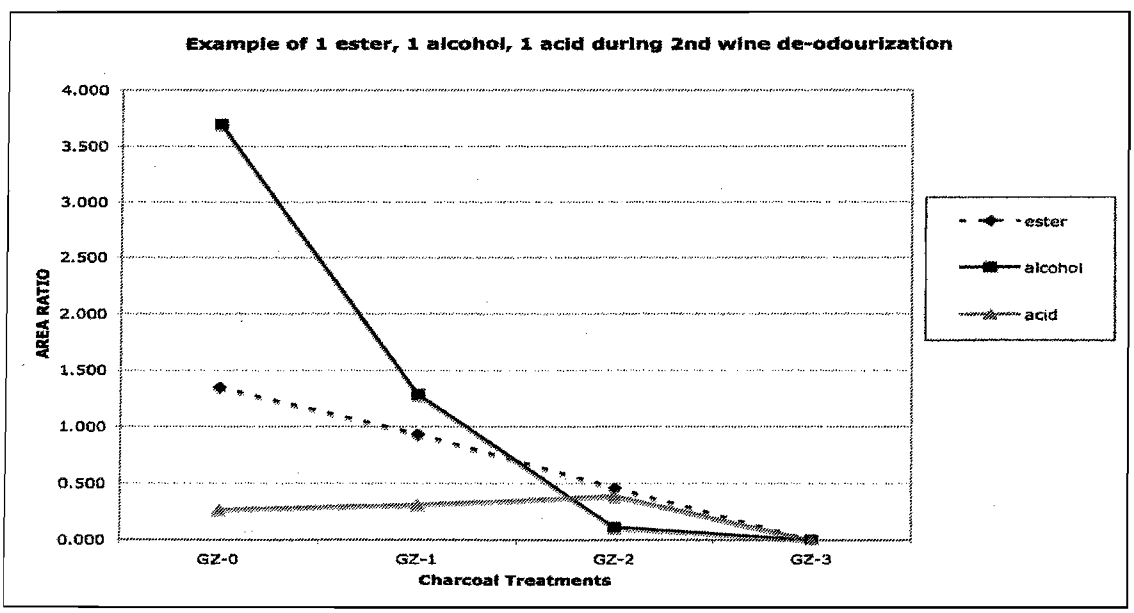


Figure A-5: Verification of volatile concentration decrease during wine deodorization process. Data represent mean values of duplicate standards ±SD

Appendix B – Preliminary Trial

Remediation of wine with elevated concentrations of 3-alkyl-2-methoxyprazines using cork and synthetic closures

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Introduction

Overall wine quality is greatly affected by volatile constituents, those that influence aroma. 3-alkyl-2-methoxypyrazines (MPs; ***Figure B-1***) are unique odor-active chemicals that are pervasive in nature (1), and wines. 3-isopropyl-2-methoxypyrazine (IPMP), 3-*sec*butyl-2-methoxypyrazine (SBMP) and 3-isobutyl-2-methoxypyrazine (IBMP) are secondary metabolites that are most abundant in grape varieties from the Bordeaux region in France (2-4) and cause significant influence on resultant wine aromas due to aromatically potent nature. MP thresholds have been reported as low as 320 pg/L (5). Although often present in very low quantities and able to contribute to pleasant varietal character (6, 7), at or above certain levels, MPs are responsible for pungent, vegetative aromas detrimental to wine quality (4, 8). MPs undergo degradation, although the mechanism remains unclear, in grapes during ripening stages of growth (9, 10) and hence are relatively elevated in under-ripe fruit (2, 4, 9, 11) and in cooler climate grapes (11, 12). Recently a secondary, exogenous source of MPs in wine has been discovered. Elevated MPs in wine can occur from the haemolymph of the lady beetle *Harmonia axyridis* (Coleoptera: Coccinellidae; *HA*), when they are unintentionally harvested with grapes and enter the fermentation process (13). IPMP excretion or extraction (13) produces atypical MP percepts in affected wines, referred to as ladybug taint (LBT; (14)). *HA* over-wintering and aggregating behaviour (15), means great quantities of beetles may be found in vineyards at a time that overlaps with commercial grape harvest (16). This has been observed in Southern Ontario in recent years and as *HA* are found in many winemaking regions of the world, including France, Italy, South America, and Spain (17), this exogenous source of elevated MPs in wine may become a global concern

Several oenological techniques, used to optimize wine sensory characteristics, have been tested for ability to alter MPs in wine. MP levels are reduced by pre-fermentation settling of white wines (18) and thermo-vinification of red wines (19). During alcoholic fermentation, MPs are stable (20, 21) and could even increase in concentration, as some commercial yeast strains may produce MPs (22). In wine, MPs are largely resilient to post-fermentation fining practices (23). Thus, novel approaches to remediation of high MP wines have been advocated.

In most wine jurisdictions, permitted additions to and treatment of wine are very limited by the applicable food and wine regulations. Thus, novel application of products and/or processes already approved for use offer one approach for mediating high MP concentrations in wine. Cork and synthetic cork closures have been shown during normal wine aging to have an affinity for sorption of some trace wine compounds (24), including MPs (25). This presents opportunities for using cork/synthetic cork, or their constituents, as function agents prior to bottling, such as passing MP-rich wine through a column consisting of these materials. The migration of odor-active compounds into packaging material is termed flavour scavenging, and has been documented in the food industry along with the reverse process, where volatile constituents may migrate from the packaging into the foodstuff (26).

In general, flavour scavenging is most pronounced between non-polar volatiles in liquid matrices and polymer packaging (27), and has been investigated with the goal of improving the flavour profile of foodstuffs by removal of undesired compounds (26). Natural wine corks contain a homogenous tissue of hexagonal cells composed of the plant polymeric compounds suberin, lignin and cellulose (28), while synthetic corks, originally produced from polyethylene (29), are now made of several polymeric, plastic materials (30). Due to material structure, non-polar or hydrophobic compounds can permeate through natural (24) and synthetic cork (30) closures. Flavour scavenging by wine closures was first reported in wine in 1999 with sorption of chloroanisoles by natural corks (24), and has been confirmed in subsequent studies for a range of odorants (31). In general, synthetic closures show greater sorptive capacity than natural corks, and the effects of flavour scavenging are more pronounced with non-polar compounds (30-32). However, relatively polar volatiles (3-mercaptohexyl acetate and 3-mercaptohexanol) have also been shown to have sorptive affinity for natural cork wine closures (32). To our knowledge, no research appears in the peer-reviewed literature to date that examines the sorptive capacity of wine closures for MPs.

Thus, the objective of this study was to determine the MP-binding behaviour of common cork products used in the industry. This information is necessary to assist in the design of potential cork-based remediation strategies, and may also inform questions regarding the optimum closure to use at bottling with wines of high MP concentration.

Materials and Methods

Preparation of wine

Chardonnay grapes from the Niagara Peninsula were sourced and juiced using commercial protocols to achieve 85L of juice. Basic juice composition (determined after (33)) was: soluble solids (°Brix): 18.2; titratable acidity (g/L): 4.8 and pH: 3.38. The following additions were then made to optimize the wine composition for fermentation (34): diammonium phosphate (to achieve 250 mgN/L), dextrose (to achieve 22 °Brix) and tartaric acid (to achieve 6.3 g/L). 55L of juice were then transferred to an American oak barrel and 30L to glass carboys (10L and 20L) and inoculated with *Saccharomyces cerevisiae* yeast strain, EC 1118 (Lallemand Inc., Santa Rosa, CA) as per manufacturer's instructions. Wines were then fermented to dryness at room temperature (barrel and 10L carboy) or 16°C (20L carboy). All wines were then inoculated with Enoferm Alpha malolactic fermentation (MLF) culture (Lallemand Inc., 1620 Prefontaine, Montreal, QC) as per manufacturer's instructions, and monitored for L-Malic acid using Megazyme® Enzyme kit (Wicklow, Ireland). After completion of MLF, wines were racked, transferred to glass carboys, sulphited (40mg/L SO_2) and stored at -2°C until cold stabilized. They were then racked, blended, sulphited (35mg/L SO_2), filtered (1 micron pad filter followed by 0.45 micron filter), bottled and stored in a wine cellar until required.

Basic quality parameters were determined on the finished wine in duplicate according to Iland *et al.* (2004). Mean values ± SD were: pH: 3.39 ± 0.0; titratable acidity (g/L): 7.05 ± 0.0; residual sugar (g/L): 2.35 ± 0.04 and total SO_2 (mg/L): 25.6 ± 1.1. Ethanol was quantified using Gas Chromatography (GC) coupled to flame ionisation detection after Nurgel et al. (2004), and was 12.1 % (v/v) ± 0.1.

Materials and protocol for soaking trials

Samples of the following commonly used commercial wine closures were sourced: Sterisun® natural corks UFB (Scott Laboratories, Petaluma, CA; 'Natural cork'), agglomerate composite corks (Scott Laboratories; 'Agglomerate cork'), Nomacorc Classic® synthetic corks (Funk Winemaking Supplies, Ontario, Canada; 'Synthetic cork – extruded') and Supreme Corq® synthetic corks (Kent, WA; 'Synthetic cork – moulded'). MP stock solutions (1mg/ml) of IPMP (97%), IBMP (99%), and SBMP (97%) (all Sigma-Aldrich, Oakville, ON) were prepared in 95% ethanol (RDL Alcohols, Grimsby, ON). Further dilutions were made with purified water (Milli-RO®; Millipore, Bedford, MA) to obtain stock concentrations of 96.6 μg/L IPMP, 98.6 μg/L SBMP and 98.0 μg/L IBMP.

From these stock solutions, 40ng/L each of IPMP, SBMP and IBMP were added to 5L of the Chardonnay base wine described above. The wine was covered to protect from light and stirred on a stir plate for approximately 18hrs. Closures were then added to 500ml Schott bottles at either 5 or 10 units/bottle and the bottles filled to overflow with the MP-enriched wine. Samples were then sealed with their respective plastic screw caps, covered, and stored in a fume hood for 140hrs at ambient room temperature (~21°C) prior to decanting off the closures and analysis. Two control wines were included: Control A - sample processed as above without addition of corks and stored in 500ml volumetric flask closed with glass stopper; Control B – sample processed as above without addition of corks and stored in 500ml Schott bottle closed with plastic screw cap. Controls wines were included to account for simple volatilization and the potential sorption of MPs onto the plastic Schott bottle caps.

Determination of methoxypyrazines and data analysis

Sample were prepared by solid-phase extraction, washing cartridges (3 mL containing 100 mg of end-capped C18 bonded porous silica, Diagnostix, Oakville, Ontario) with 0.5 mL of ethyl acetate followed by 0.5 mL of 95% v/v ethanol and finally with 0.5 mL of 10% v/v ethanol. 25 mL of the wine sample was passed through the cartridge, which was then dried for 10 mins using a pump (20mm mercury) vacuum. The absorbed MPs were eluted by adding 0.4 mL of dichloromethane and eluent collected in a calibrated vial made up to 0.5 mL with ethyl acetate.

A GC coupled to a Mass Selective Detector (MSD; Agilent 5890 GC and 5973 MSD; Agilent, Mississauga, Ontario) was fitted with a DB-5 MS column (30 m long x 0.25 mm I.D., 0.25 μm thickness; J&W Scientific, Folsom, CA). A He carrier gas flow of 1 mL/min was used. Temperature programming was as follows: initial temperature 70°C, held for 0.5 min; increased 10°C/min to 230°C, held for 0.1 min; increased 30°C/min to 250°C, held for 2 mins. Injector and detector temperatures were 200°C and 230°C, respectively. 2 μL of sample was injected in splitless mode. The MSD operated in selective ion monitoring mode and each compound was quantitated based on response of peak area using different ions: m/z 124, 137 and 152 for IPMP, m/z 124 and 151 for IBMP, and m/z 151 and 138 for SBMP. Concentration of the compounds was determined by an external standard using a three-point calibration curve. Calibration standards were prepared by dilution of stock standard solution in a MP-free wine extract. The calibration standards were compared against a separate standard made up in ethyl acetate. Fresh standards were prepared for each analysis run. Duplicate analysis of samples was performed.

Data were analyzed using the ANOVA procedure within XLSTAT© version 7.5.2 (Addinsoft, 40, rue Damrémont, 75018 Paris, France). If p(F) was <0.05, Fisher's LSD was used as the means separation test.

Results and Discussion

Key findings

3-alkyl-2-methoxpyrazine (MP) concentrations are displayed in ***Figure B-2***. MPs decreased after the soaking period for each closure treatment compared to control bottles. Interestingly, MP concentrations were 5-10% lower in wine from Control B compared to Control A, indicating the sorptive capacity of the plastic Schott bottle tops used in the treatments. This loss is accounted for in the calculations and discussion that follows.

Synthetic corks affected MPs the greatest, with decreases up to 70 %, 77 % and 89 % for IPMP, IBMP and SBMP, respectively. Natural and agglomerate cork also show some and similar sorptive capacities, except for the greater decrease observed with Agglomerate-10 for SBMP. As expected, loss of MPs was greater in 10-cork treatments compared to 5-cork treatments for each closure type, with the exception of Agglomerate (IBMP), Synthetic-moulded (IBMP) and Natural (SBMP).

As the closures vary in surface area, we also calculated the sorption efficiency of the closure material as a function of surface area (***Table B-1***). The same major trends noted above are observed. Of the 3 MPs, SBMP decreases the most, regardless of closure type or number, indicating its greater sorptive properties. Odour detection thresholds can provide an indication of the aroma-potency of these vegetative compounds. Detection thresholds for IPMP and IBMP in wine are in the 0.3-10ng/L range (5, 11, 36) and, based on thresholds in water, likely similar for SPMP (37). Difference thresholds have not been determined for MPs in wine, however, we speculate that due to the relatively large concentration differences observed, many of these treatments would be perceptibly less vegetative than the Control wines. Indeed, sensory profiling of wines treated in this manner would be of value; volume restrictions prevented this in the current study.

Other considerations and further research

Synthetic closures appear to have the greatest potential for use in remediation of MPs, given their superior performance in this trial, and the known propensity for natural cork-based closures to contribute undesirable odorants, such as 2,4,6-trichloroanisole (24), into wine. Given restrictions in permitted additives to and treatment of wines, one approach could be to pass wine through a column that uses synthetic closures, or smaller polyethylene-based materials, as the stationary phase. This may require only minor modification of equipment already in use in the wine industry, such as ion-exchange columns. Further research concerning such a scheme should also determine the potential removal of non-target wine impact odorants and anthocyanins, and the consequences on quality.

Alternatively, more passive scavenging of MPs may be possible through normal bottle-aging when synthetic closures are employed. Further trials are needed to monitor changes in MP content in wines exposed to a range of closure types under ecologically-valid wine aging/cellaring conditions. The significantly reduced surface area offered by a single closure could be a major limitation, but presents opportunities for closure manufacturers to optimize the design and functionality of their products to meet growing demands to control or modify wine flavor post-bottling.

Conclusion

Natural cork, agglomerate cork and synthetic corks (both extruded and moulded) all demonstrate sorptive capacity for MPs. SBMP is affected the most by treatment with these closures, and soaking with either synthetic cork resulted in significantly greater reduction in MP concentrations than soaking with natural cork-based closures. These results indicate that synthetic, polyethylene-based wine closures have the potential to remediate wines of elevated MP content both pre- and post-bottling.

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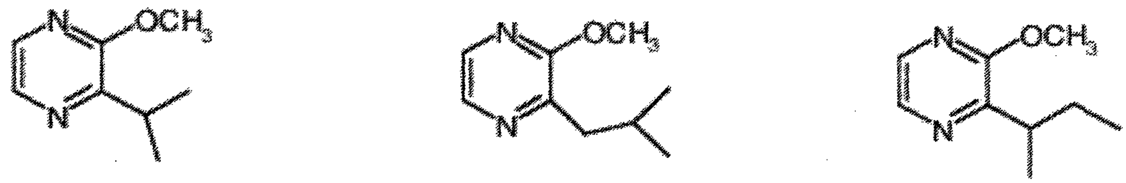
Figures



IPMP IBMP SBMP

Figure B-1 – 3-alkyl-2-methoxypyrazines identified in grape and wine: 3-isopropyl-2-methoxypyrazine (IPMP), 3-isobutyl-2-methoxypyrazine (IBMP) and 3-*sec*butyl-2-methoxypyrazine (SBMP)

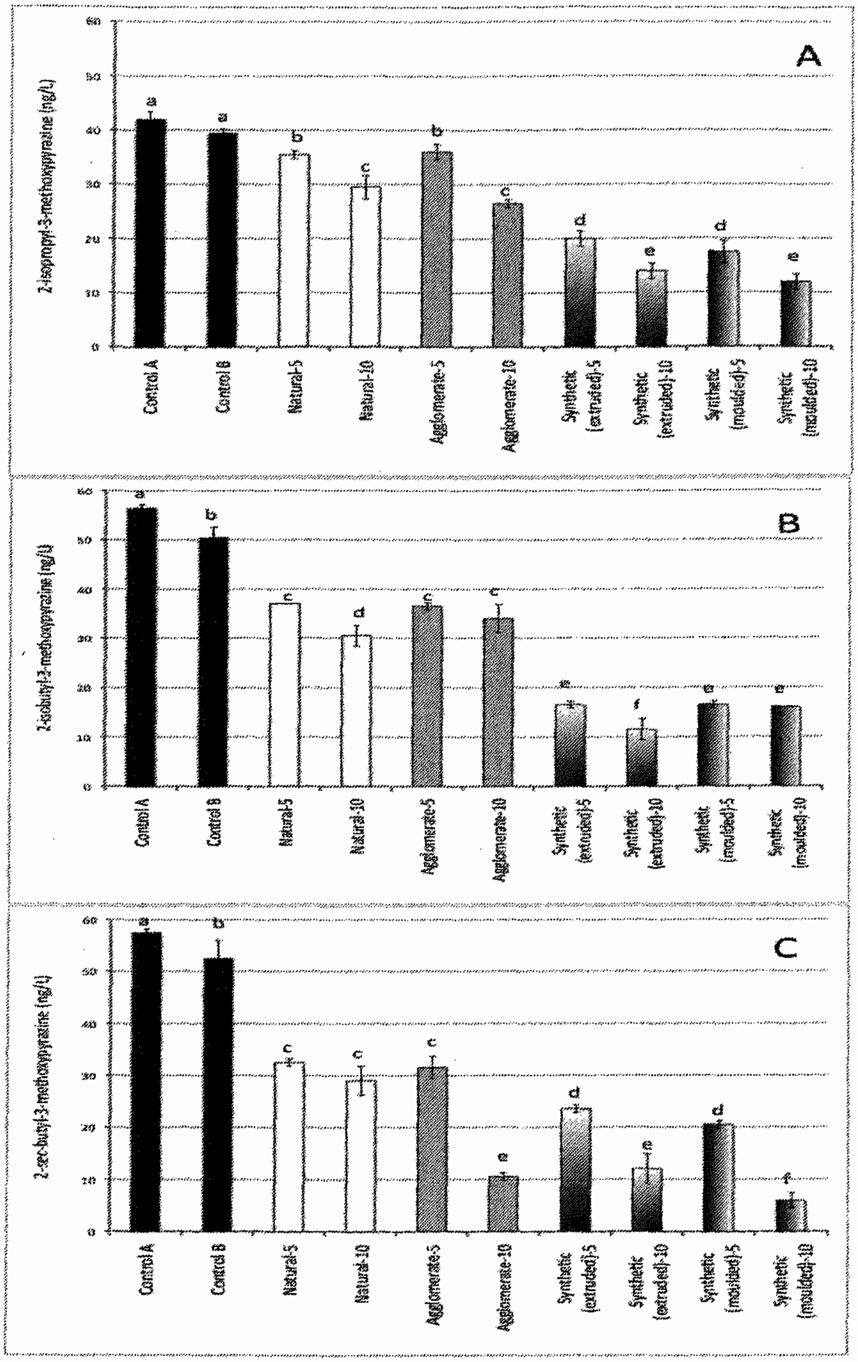


Figure B-2 - Concentration of isopropyl- (A), isobutyl- (B) and *sec*-butyl- (C) methoxypyrazine (MP) in a MP-enriched Chardonnay wine after soaking with various cork products for 140 hrs Data represent mean values ± 1 SD. Different letters represent treatments with different means (Fisher's Protected LSD, p>0.05). Control A was stoppered with glass. Control B was stoppered with plastic Schott bottle top used in cork treatments

Table

Table B-1 - Sorption efficiencies of various wine closures for 3-alkyl-2-methoxypyrazines [a]

	Natural cork [b]	*Agglomerate cork* [c]	*Synthetic cork (extruded)* [d]	*Synthetic cork (moulded)* [e]
Methoxypyrazine				
	5-cork treatment			
isopropyl-	0.02	0.02	0.11	0.16
isobutyl-	0.08	0.09	0.19	0.24
sec-butyl-	0.11	0.13	0.16	0.23
	10-cork treatment			
isopropyl-	0.03	0.04	0.07	0.10
isobutyl-	0.06	0.05	0.11	0.12
sec-butyl-	0.07	0.13	0.11	0.16

a: data represent decrease in methoxypyrazine concentration (ng/L) in a Chardonnay wine after addition of closures for 140 hrs /surface area of the closures (cm^2), adjusted for wine volume; methoxypyrazine values based on average of duplicate measurements; b: Sterisun UFB; c: Agglomerate composite corks (Scott Labs); d: Nomacorc Classic; e: Supremecorq